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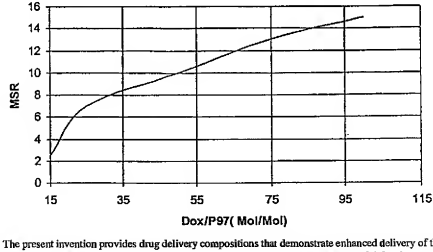
(54) Title: COMPOSITIONS OF COMPOUNDS CONJUGATED TO P97 AND THEIR METHODS OF USE



(57) Abstract: The present invention provides drug delivery compositions that demonstrate enhanced delivery of therapeutic agents to selected organs, in particular to non-CNS organs. The compositions of the invention are useful for the treatment of diseases associated with these organs. In addition, the compositions of the invention reduce the systemic toxicity of the compounds. In certain aspects, the pharmaceutical compositions comprise a compound conjugated to p97 or to a fragment thereof, and a pharmaceutically acceptable carrier therefor.

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COMPOSITIONS OF COMPOUNDS CONJUGATED TO P97 AND THEIR METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application No. 60/226,242, filed August 17, 2000, which is hereby incorporated by reference for all purposes. U.S. Patent Application No. 60/226,254, filed August 17, 2000, is hereby incorporated by reference for all purposes.

FIELD OF THE INVENTION

The present invention relates to drug delivery compositions for enhanced delivery of therapeutic agents to selected organs, for treatment of diseases associated with these organs, and for reducing the systemic toxicity of therapeutic agents.

BACKGROUND OF THE INVENTION

p97, also known as melanotransferrin (or Mtf), is a human melanoma-associated protein antigen. It was one of the first cell surface markers to be associated with human skin cancer (Hellstrom and Hellstrom (1982) in *Melanoma Antigens and Antibodies*, Ed. Reisfield and Ferrone, Plenum Press, NY, pp. 187-341). p97 is a monomeric membrane-associated protein with a molecular mass of 97,000 daltons (Brown *et al.* (1981) *J. Immunol.* 127:539) and has been suggested as a melanoma specific marker (Estin *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:1052-1056). As well as being associated with the cell surface of melanomas and some other tumors and cell lines (Brown *et al.* (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78:539), p97 has also been found in certain fetal tissue (Woodbury, *et al.* (1981) *Int. J. Cancer* 27:145) and, more recently, on endothelial cells of the human liver (Sciot *et al.* (1989) *Liver* 9:110). Homologs of p97 have now been identified in other animal species such as mouse, chicken, pig and rabbit.

The primary structure of p97, deduced from its mRNA sequence, indicates that it belongs to a group of closely related iron binding proteins found in vertebrates (Rose *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:1261). This family includes serum transferrin, lactoferrin and avian egg white ovotransferrin. Human p97 and lactoferrin share 40% sequence homology (Baker *et al.* (1987) *Trends Biochem. Sci.* 12:350), however, in contrast to the other molecules of the transferrin family, p97 is the only one

which is directly associated with the cell membrane. The deduced sequence of p97 has, in addition to a transferrin-like domain, a hydrophobic segment at its C-terminus. This hydrophobic C terminus is cleaved post-translationally. A glycosyl phosphatidyl inositol is attached to p97 to generate the predominant membrane bound form of the mature molecule (Food *et al.* (1994) *J. Biol. Chem.* 269(4):3034-3040).

Published work on p97 has most recently focused on its possible physiological roles as a diagnostic indicator of Alzheimer's disease, and a highly selective transporter of iron across the blood-brain barrier (*see, e.g.*, Kennard *et al.* (1996) *Nat. Med.* 2(11):1230-1235; and Yamada *et al.* (1999) *Brain Res.* 845:1-5).

It is an object of the instant invention to provide, for the first time, new compositions of p97-linked compounds which achieve therapeutic, prophylactic and diagnostic objectives in organs distinct from the brain, such as the liver, lung, kidney and spleen. In addition, these new compositions reduce the cardiotoxicity of cardiotoxic drugs and increase the maximum tolerated dose of such drugs.

SUMMARY OF THE INVENTION

The present invention is directed to the discovery that p97-compound compositions, in which a compound of interest is covalently linked to p97 or to a fragment thereof, are excellent vehicles for the enhanced delivery of the compound to selected organs. Such p97-compound compositions are useful, *e.g.*, for the treatment of diseases associated with an organ of interest, for reducing the systemic toxicity of therapeutic agents, and, in particular, for reducing the cardiotoxicity of cardiotoxic drugs, and for increasing the maximum tolerated dose of such cardiotoxic compounds.

Preferred compositions comprise from about 1 to about 20 molecules of a compound of interest linked to a single p97 molecule to form a p97-compound. Selective biodistribution of p97-compounds enhances selective targeting of p97-linked compounds to specific organs.

Further preferred p97-compounds are p97-chemotherapeutic agent compositions. These compositions provide improved delivery of chemotherapeutic agents to organs including, but not limited to, the liver, lung, spleen, and kidney, for the treatment of disorders associated with those organs, such as malignant neoplasia, for reducing the cardiotoxicity of cardiotoxic chemotherapeutic agents, and for increasing the maximum tolerated dose of such cardiotoxic chemotherapeutic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of increasing molar excess of the activated therapeutic agent (*e.g.*, ADR) on the effective ratio of p97:ADR linking. Increasing the amount of ADR in the reaction mixture increases the Molecular Substitution Ratio (MSR).

Figure 2 shows the tissue/serum ratio of p97-I^{125} (Apo and holo) versus BSA- I^{125} at 1 hour post-intravenous injection.

Figure 3 illustrates the relative % increase in uptake of p97-I^{125} (Apo and holo) versus uptake of BSA- I^{125} at 15 minutes after administration.

Figures 4A and 4B illustrate the relative % increase in uptake of p97-I^{125} versus uptake of BSA- I^{125}. Figure 4A illustrates the relative % increase in uptake of p97-I^{125} (Apo and holo) versus uptake of BSA- I^{125} at 1 hour after administration. Figure 4B illustrates the distribution of p97-I^{125} versus uptake of BSA- I^{125} in 13-day C6 ic tumor 2hr post 1x3.4 Mcpm (4.3 μg) p97 or 3 Mcpm BSA (n = 1).

Figure 5 shows a comparison of tissue distribution of p97-ADR and free ADR at 1 hour after administration.

Figure 6 shows a comparison of uptake of p97-ADR and free ADR by heart tissue

Figure 7 shows the effect of ADR and p97-Adr by intravenous administration on tumor inhibition.

Figure 8 shows the effect of ADR and p97-ADR by intravenous administration on tumor inhibition at day 21.

Figure 9 illustrates the growth inhibition of a subcutaneous C6 glioma tumor by p97-Adr administered intraperitoneally.

Figure 10 shows a composite electromicrograph demonstrating the specific uptake vesicles containing p97-gold in liver tissue samples.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

I. INTRODUCTION

The present invention provides compositions and methods for effectively delivering compounds to the liver, lung, spleen, kidney, and to other organs, for the treatment of a variety of disorders associated with those organs, such as malignant neoplasia, for reducing the systemic toxicity of therapeutic agents, and in particular for

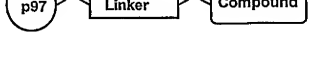
reducing the cardiotoxicity of cardiotoxic chemotherapeutic agents, and for increasing the maximum tolerated dose of such cardiotoxic chemotherapeutic agents. Enhanced delivery to specific organs is achieved by linking the compounds directly to p97 or a to a fragment thereof. Preferred compositions comprise from about 1 to about 20 molecules of the compound linked to each p97 molecule or fragment thereof. A wide range of compositions are provided which are useful for treating a variety of disorders in mammals, in particular in humans.

II. DEFINITIONS

The term "p97" as used in the compositions of the invention, includes membrane bound p97 (*i.e.*, p97 linked to GPI or other lipids), soluble p97, cleaved p97, analogs of p97 (having greater than 40% homology at the peptide sequence level, including allelic variants of p97), human, mouse, chicken and/or rabbit p97, and derivatives, portions, or fragments thereof. p97 may be in the form of acidic or basic salts, or in neutral form. In addition, individual amino acid residues may be modified, such as by oxidation or reduction. Various substitutions, deletions, or additions may be made to the amino acid or DNA nucleic acid sequences, the net effect of which is to retain or improve upon the desired biological activity of p97. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

A "p97 fragment," as used herein, refers to any portion of p97, or its biologically equivalent analogs, that contains a sufficient portion of p97 to enable it to be transported across the blood brain barrier; or that otherwise retains or improves upon the desired biological activities of p97.

A "p97-compound" refers to a composition comprising p97, or a fragment thereof, covalently conjugated to another compound. The conjugation may be direct or indirect (*i.e.*, through an extended linker) so long as it is a chemical conjugation. The general construct of the composition of the invention is as follows:



"Therapeutic, prophylactic and diagnostic agents" as used herein refers to agents which are suitable for achieving these objectives in animals, preferably in mammals.

Preferred compounds for use in the p97-compounds of the invention include all drugs which may be relevant for treating disorders found at the non-CNS p97 target organs such as the lung, liver, kidney and spleen. A typical disorder is a neoplasia. Drugs for treating neoplasia include, for example, adriamycin (doxorubicin), cisplatin, taxol, taxotere and other chemotherapeutic agents which demonstrate activity against tumours *ex vivo* and *in vivo*.

Preferred compounds for treating infections at the p97 target organs include, *e.g.*, antibiotics, antiinfective agents, such as amphotericin B and anti-fungal agents and the like, *etc.*

Preferred compounds for use for treating non-CNS organs include, but are not limited to, all small molecules, biologics, nucleic acids and modifications of these compounds. Peptide hormones such as insulin, somatostatin and the like are useful compounds. Anti-angiogenic compounds, such as angiostatin, endostatin and the like, are useful because of the ability of p97 to deliver such compounds preferentially to certain tumor types. Because the compound must be conjugated to p97, it is preferable to use an agent containing an alcohol, acid, carbonyl, thiol or amine group in the conjugation to p97. Linkage may also be achieved through a carbonyl group, such as, for example, in the case of an amine, such as Adriamycin. Taxol and morphine are both alcohols and are also suitable for use in the compositions of the present invention. Compounds without suitable conjugation groups may be further modified to add such a group. All these compounds are contemplated in this invention.

Certain compounds which have both therapeutic and diagnostic uses as p97-compounds also include base elements and metals, fluorescent molecules, toxins, substances having therapeutic activity, including, but not limited to, therapeutic compounds, luminescent molecules, enzymes, and radionuclides. Representative examples of fluorescent and luminescent molecules include fluorescein, phycoerythrin, rodamine, Texas red; luciferase, Cy5.0, Alexa Fluor™ (Molecular Probes, Inc.). Representative examples of base elements, metals, some of which may be radionuclides, include, for example, Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212 gold, iron (Fe-55; Fe-59), zinc, aluminum, magnesium, manganese, and the like.

Preferred compounds include therapeutic agents which in the free form demonstrate unacceptable systemic toxicity at the desired doses. The general systemic toxicity of these agents is reduced by linkage to p97. Particularly preferred are cardiotoxic compounds which are useful therapeutics but are dose limited by cardiotoxicity. A classic example is adriamycin (also known as doxorubicin) and its analogs, such as daunorubicin. Linking p97 to such drugs effectively prevents the accumulation in the heart and associated cardiotoxicity.

"Increasing relative delivery" as used herein refers to the effect whereby the accumulation at a site (such as an organ or a neoplasia) of a modified composition is increased relative to the accumulation of the original composition. This may be caused by increased specific or non-specific binding of the modified composition at the site of interest compared to the original composition.

"Therapeutic index" refers to the dose range (amount and/or timing) above the minimum therapeutic amount and below an unacceptably toxic amount.

"Equivalent dose" refers to a dose which contains the same amount of active agent.

"Unacceptable cardiotoxicity" refers to a level of cardiotoxicity which is deemed unacceptable by a skilled analyst, and may vary depending on the patient.

II. COMPOSITIONS AND PREPARATION THEREOF

The present invention generally provides methods and compositions for enhanced delivery of the above-described compounds to selected organs, for the treatment of diseases associated with these organs, for reducing the cardiotoxicity of cardiotoxic compounds, and for increasing the maximum tolerated dose of such cardiotoxic compounds.

In general, p97-compounds may be prepared using techniques well known in the art. There are numerous approaches for the conjugation or chemical crosslinking of compounds to a peptide such as p97, and one skilled in the art can determine which method is most appropriate for conjugating a particular compound. The method employed must be capable of joining the compound with p97 without interfering with the ability of p97 to bind to its receptor, preferably without influencing the biodistribution of the p97-compound compared to p97 alone, and/or without significantly altering the desired activity of the compound (be it therapeutic, prophylactic, diagnostic or the like) once delivered. Preferred methods of conjugating p97 to various compounds are

described in the examples section, below. A particularly preferred method for linking complex molecules to p97 is the SATA/sulfo-SMCC cross-linking reaction (Pierce (Rockford, IL)). For linking metals to p97, preferred reactions include binding to tyrosine residues through Chloramine T methods, or use of Iodo beads (Pierce) for iodination reactions. Such methods are well known to those of skill in the art.

Methods for conjugating or labeling p97 with the representative labels described above may be readily accomplished by one of ordinary skill in the art (*see, e.g.*, Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; *see* also Inman, *Methods In Enzymology*, Vol. 34, *Affinity Techniques, Enzyme Purification*: Part B, Jakoby and Wichek (eds.), Academic Press, NY, p. 30 (1974); *see* also Wilchek and Bayer (1988) "*The Avidin-Biotin Complex in Bioanalytical Applications*" *Anal. Biochem.* **171**:1-32).

For techniques employing p97-Fe, the normal iron binding characteristic of p97 may be used without chemical conjugation. A standard iron loading protocol for producing holo p97 is described below. Apo p97 (p97 free of iron) may be generated with a standard dialysis technique, also described below.

When the compound is a protein or a peptide, several hundred crosslinkers are available for conjugating a compound of interest with p97 or with a substance which binds p97 (*see, e.g., Chemistry of Protein Conjugation and Crosslinking*, Shans Wong, CRC Press, Ann Arbor (1991)). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the therapeutic compound. In addition, if there are no reactive groups, a photoactivatible crosslinker can be used. In certain instances, it may be desirable to include a spacer between p97 and the compound. In one embodiment, p97 and the protein therapeutic compounds may be conjugated by the introduction of a sulfhydryl group on p97 and by the introduction of a crosslinker containing a reactive thiol group on to the protein compound through carboxyl groups (Wawizynczak and Thorpe in *Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer*, Vogel (Ed.) Oxford University Press, pp. 28-55 (1987); and Blair and Ghose (1983) *J. Immunol. Methods* 59:129).

In some embodiments of the present invention, the p97-compound is a p97-fusion protein. Fusion proteins may be prepared using standard techniques known in

the art. Typically, a DNA molecule encoding p97 or a portion thereof is linked to a DNA molecule encoding the protein compound. The chimeric DNA construct, along with suitable regulatory elements can be cloned into an expression vector and expressed in a suitable host. The resultant fusion proteins contain p97 or a portion thereof used to the selected protein compound. Examples of proteins which may be used to prepare the fusion proteins of the invention include, for example, lymphokines such as gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1, TNF, NGF and G-CSF.

In other embodiments, the p97-compounds may comprise one or more compound moieties linked to p97. For example, conjugation reactions may conjugate from 1 to about 10 or more molecules of adriamycin to a single p97 molecule. Several atoms of gold or iodine may be conjugated to a single p97 polypeptide. These formulations may be employed as mixtures, or they may be purified into specific p97:compound (mol:mol) formulations. Those skilled in the art can easily determine which format and which mol:mol ratio is preferred. Furthermore, mixtures of compounds may be linked to p97, such as the p97-adriamycin-cisplatinum composition described herein. These p97-compounds may consist of a range of mol:mol ratios. These, too, may be separated into purified mixtures or they may be employed as aggregates.

When a linker is used, the linker is preferably an organic moiety constructed to contain an alkyl, aryl and/or amino acid backbone, and containing an amide, ether, ester, hydrazone, disulphide linkage or any combination thereof. Linkages containing amino acid, ether and amide bound components are stable under conditions of physiological pH, normally 7.4 in serum and 4-5 upon uptake into cells (endosomes). Preferred linkages are those containing esters or hydrazones that are stable at serum pH but that hydrolyze to release the drug when exposed to intracellular pH. Disulphide linkages are preferred because they are sensitive to reductive cleavage. In addition, amino acid linkers may be designed to be sensitive to cleavage by specific enzymes in the desired target organ. Exemplary linkers are described in Blattler *et al.* (1985) *Biochem.* 24:1517-1524; King *et al.* (1986) *Biochem.* 25:5774-5779; Srinivasachar and Nevill (1989) *Biochem.* 28:2501-2509.

Drug-Linker intermediates are similar to what has been described previously, but comprise either an active ester that can react with free amine groups on p97 or a maleimide that can react with the free thiols created on p97 via a SATA reaction or through other groups where persons skilled in the art can attach them to p97.

A. Preparation of p97

The p97 peptide for use in the methods and compositions of the present invention may be obtained, isolated or prepared from a variety of sources.

In one aspect, standard recombinant DNA techniques may be used to prepare p97 or derivatives thereof. Within one embodiment, DNA encoding p97 may be obtained by polymerase chain reaction (PCR) amplification of the p97 sequence (*see*, generally, U.S. Patent Nos. 4,683,202; 4,683,195; and 4,800,159; *see*, also, *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press (1989)). Briefly, double-stranded DNA from cells which express p97 (*e.g.*, SK-MEL-28 cells) is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers such as 5' GCGGACTTCCTCGG 3' (SEQ ID NO:1) and 5' TCGCGAGCTTCCT 3' (SEQ ID NO:2), ATP, CTP, GTP and TTP. Double-stranded DNA is produced when the synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of p97 DNA. The amplified p97 DNA may then be readily inserted into an expression vector as described below.

Alternatively, DNA encoding p97 may be isolated using the cloning techniques described by Brown *et al.* in the UK Patent Application No. GB 2188 637. Clones which contain sequences encoding p97 cDNA have been deposited with the American Type Culture Collection (ATCC) under deposit numbers CRL 8985 (PMTp97b) and CRL 9304 (pSVp97a).

Within one embodiment of the present invention, truncated derivatives of p97 are provided. For example, site-directed mutagenesis may be performed with the oligonucleotide WJ31 5'CTCAGAGGGCCGCTGCGCCC-3'(SEQ ID NO:3) in order to delete the C-terminal hydrophobic domain beyond nucleotide 2219, or with the oligonucleotide WJ32 5' CCA GCG CAG CTAGCGGGGGCAG 3' (SEQ ID NO:4) in order to introduce an Nhe I site and a STOP codon in the region of nucleotides 1146-1166, and thereby also constructing a truncated form of p97 comprising only the N-terminal domain. Similarly, mutagenesis may also be performed on p97 such that only the C-terminal domain is expressed. Within one embodiment, Xho sites are inserted by mutagenesis with the oligonucleotide WJ 5'-ACACCAGCGCAGCTCGAGGGGCAGCCG 3' (SEQ ID NO:5) into both the N-terminal and C-terminal domains, allowing subsequent deletion of the N-terminal domain. Various other restriction enzymes, including for example, Eco RI, may also be utilized in the context of the present invention in order to construct deletion or truncation derivatives of p97.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling the ligation of the mutated fragments to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion. Alternatively, as noted above oligonucleotide-directed site-specific mutagenesis procedures may be employed to obtain an altered gene having particular codons altered according to the desired substitution, deletion, or insertion. Exemplary methods of making the alterations set forth above are disclosed by Sambrook *et al. Molecular Cloning A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press (1989).

Within a particularly preferred embodiment of the invention, p97 is cloned into an expression vector as a truncated cDNA with a deletion of the GPI anchor sequence located in the carboxy terminus of the protein.

Briefly, the p97 gene is generated by polymerase chain reaction (PCR) using the cloned p97 cDNA as a template. The truncated p97 is synthesized using WJ47, the 5' PCR primer encompassing coordinates 36 to 60 (coordinates based on the cDNA map) and additionally containing a Sna BI restriction site. The sequence of WJ47 is 5'-GCG CTA CGT ACT CGA GGC CCC AGC CAG CCC CGA CGG CGC C-3' (Seq ID:6). The 3' primer, WJ48, encompasses coordinates 2172 to 2193 and additionally contains both a TGA termination codon and a SnaBI restriction site. The DNA sequence of WJ48 is 5'-CGC GTA CGT ATG ATC ATC AGC CCG AGC ACT GCT GAG ACG AC-3' (Seq ID:7). Following amplification, the truncated p97 product is inserted into pNUTAH (obtained from Palmiter (1986) *PNAS* 83:1261-1265) at the Sma I restriction site. The orientations of the resulting plasmids may be determined by PCR using one priming oligonucleotide which anneals to the vector sequence and a second priming oligonucleotide which anneals to the insert sequence. Alternatively, appropriate restriction digests can be performed to verify the orientation. Expression of the amplified sequence results in the production of a soluble p97 protein lacking the hydrophobic domain.

As noted above, the present invention provides recombinant expression vectors which include either synthetic, or cDNA-derived DNA fragments encoding p97 or derivatives thereof, which are operably linked to suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, including, but not limited to, bacterial, fungal, viral, mammalian, and insect

genes. Selection of appropriate regulatory elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include, in particular, a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may be incorporated into the expression vector.

DNA sequences encoding p97 may be expressed by a wide variety of prokaryotic and eukaryotic host cells, including, but not limited to, bacterial, mammalian, yeast, fungi, viral, plant, and insect cells. Methods for transforming or transfecting such cells for expressing foreign DNA are well known in the art (*see, e.g.*, Itakura *et al.*, U.S. Patent No. 4,704,362; Hinnen *et al.* (1978) *PNAS USA* 75:1929-1933; Murray *et al.*, U.S. Patent No. 4,801,542; Upshall *et al.*, U.S. Patent No. 4,935,349; Hagen *et al.*, U.S. Patent No. 4,784,950; Axel *et al.*, U.S. Patent No. 4,399,216; Goeddel *et al.*, U.S. Patent No. 4,766,075; and Sambrook *et al.*, *supra*).

Promoters, terminators, and methods for introducing expression vectors of an appropriate type into, for example, plant, avian, and insect cells may be readily accomplished by those of skill in the art. Within a particularly preferred embodiment of the invention, p97 is expressed from baculoviruses (*see, e.g.*, Luckow and Summers (1988) *BioTechnology* 6:47; Atkinson *et al.* (1990) *Petic. Sci.* 28:215-224). The use of baculoviruses such as AcMNPV is particularly preferred since host insect cells express the GPI-cleaved forms of p97. p97 may be prepared from cultures of the host/vector systems described above that express the recombinant p97. Recombinantly produced p97 may be further purified as described in more detail below.

The soluble form of p97 may be prepared by culturing cells containing the soluble p97 through the log phase of the cell's growth and collecting the supernatant. Preferably, the supernatant is collected prior to the time at which the cells lose viability. Soluble p97 may then be purified as described below, in order to yield isolated soluble p97. Suitable methods for purifying the soluble p97 can be selected based on the hydrophilic property of the soluble p97. For example, the soluble p97 may be readily obtained by Triton X-114 Phase Separation.

In another example, p97 may be isolated from cultured CHO cells genetically engineered to express the GPI-anchored p97. The GPI-anchored protein may

be harvested by a brief incubation with an enzyme capable of cleaving the GPI anchor. Such enzymes are known in the art (Ferguson (1988) *Ann. Rev. Bichem.* 57:285-320) and representative examples are described *supra*. The cleaved soluble protein may be recovered from the medium, and the cells may then be returned to growth medium for further expression of the protein. Cycles of growth and harvest may be repeated until sufficient quantities of the protein are obtained. A particularly preferred GPI enzyme is phospholipase C (PI-PLC) which may be obtained either from bacterial sources (*see*, Low "*Phospholipase Purification and Quantification*" The Practical Approach Series: Cumulative Methods Index, Rickwood and Hames, eds. IRC Press, Oxford, NY (1991); Kupe *et al.* (1989) *Eur. J. Biochem.* 185:151-155; Volwerk *et al.* (1989) *J. Cell. Biochem.* 39:315-325) or from recombinant sources (Koke *et al.* (1991) *Protein Expression and Purification* 2:51-58; and Henner *et al.* (1986) *Nuc. Acids Res.* 16:10383).

p97 and derivatives thereof, including the soluble p97, may be readily purified according to the methods described herein. Briefly, p97 may be purified either from supernatants containing solubilized p97, or from cultured host/vector systems as described above. A variety of purification steps, used either alone or in combination may be utilized to purify p97. For example, supernatants obtained by solubilizing p97, or from host/vector cultures as described above, may be readily concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit, or by "salting out" the protein followed by dialysis. In addition, the supernatants or concentrates may be applied to an affinity purification matrix such as an anti-p97 antibody bound to a suitable support. Alternatively, an anion exchange resin, such as a matrix or substrate having pendant diethylaminoethyl (DEAE) groups, may be employed. Representative matrices include acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Similarly, cation exchangers which utilize various insoluble matrices such as sulfopropyl or carboxymethyl groups may be also used.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps using hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other alipathic groups, can be employed to further purify p97.

p97 fragments may also be generated using the techniques described above, with modifications well known in the art. For example, p97 expression vectors may be modified so that the expressed protein is a desired fragment of p97. This protein may be isolated from the expression system (*i.e.*, extracted from cells), or it may be

designed to be secreted into the supernatant of the expression system, and isolated using techniques described above. Alternatively, full length p97 protein may be generated and purified, and p97 fragments may then be generated by cleavage reactions designed to generate the desired fragment. Chemical synthesis is an alternative route to obtain the desired p97 protein or fragment thereof.

In the context of the present invention, "isolated" or "purified," as used to define the purity of p97, refer to a protein that is substantially free of other proteins of natural or endogenous origin, and that contains less than about 5% and preferably less than about 1% by mass of protein contaminants due to the production processes. p97 may be considered "isolated" if it is detectable as a single protein band upon SDS-PAGE, followed by staining with Coomassie Blue.

B. Preparation of Antibodies to p97

Based on the teaching of the instant specification, antibodies to mouse or human p97 have many uses including, but not limited to, the use for the isolation and purification of p97, use in research and identification of p97 both *in vitro* and *in vivo*, and potential diagnostic and therapeutic uses. It is, therefore, useful to briefly set forth preferred antibodies to p97, and methods of producing such antibodies.

Antibodies reactive against p97 are well known in the art. Additional anti-p97 antibodies are provided by the present invention. Representative examples of anti-p97 antibodies include L235 (ATCC No. HB 8466; *see*, Real *et al.* (1985) *Cancer Res.* 45:4401 4411; *see*, also, Food *et al.* (1994) *J. Biol. Chem.* 269(4): 3034-3040), 4.1, 8.2, 96.5 and 118.1 (*see*, Brown *et al.* (1981) *J. Immunol.* 127(2):539-546; and Brown *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78(1):539-543); and HybC (Kennard *et al.* (1996) *Nat. Med.* 2(11):1230-1235). Other monoclonal antibodies, including, but not limited to, 2C7 and 9B6, have been generated at Synapse Technologies Inc. Antibodies to the mouse p97 include, for example, a rabbit anti-human p97 polyclonal antibody generated against a fragment of the mouse p97. In the context of the present invention, antibodies are understood to include, for example, monoclonal antibodies, polyclonal antibodies, antibody fragments (*e.g.*, Fab, and F(ab')2) and recombinantly produced binding partners. Antibodies are understood to be reactive against p97 if the Ka is greater than or equal to 10^{-7} M.

Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals. Monoclonal antibodies may also be

readily generated using conventional techniques (*see, e.g.*, U.S. Patent Nos. RE 32,011, 4,902,614; 4,543,439; and 4,411,993; *see*, also, Kennett, McKearn, and Bechtol (eds.) *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, (1980); and Harlow and Lane (eds.) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1988)). Preparation of preferred antibodies is further described in the examples section, below.

III. METHODS OF USING COMPOSITIONS

The p97-compounds of the invention are useful for therapeutic, prophylactic and diagnostic interventions animals, and in particular in humans. As described herein, p97-compounds show preferential accumulation in the lung, liver, kidney and spleen, and that they significantly reduce delivery of the compounds to the heart. Preferred medical indications for diagnostic uses include, for example, any condition associated with a non-CNS target organ of interest (*e.g.*, lung, liver, kidney, spleen) or any condition that requires a cardiotoxic compound that would benefit by reducing its cardiotoxicity. Non-CNS target organs include, but are not limited to, brain, skin, lung, liver, kidney, spleen, gallbladder, bladder, bone marrow, tumors, *etc.*

This invention also provides methods for testing the ability of different p97-compounds to achieve delivery to a selected target organ. Further assays may be performed to determine if the desired therapeutic, prophylactic, or diagnostic effect of the compound is achieved. Suitable *in vivo* models for carrying out these assays are typically whole animals such as mice, rats, rabbits and the like.

Compositions of the present invention may be administered encapsulated in or attached to viral envelopes or vesicles, or incorporated into cells. Vesicles are micellular particles which are usually spherical and which are frequently lipidic. Liposomes are vesicles formed from a bilayer membrane. Suitable vesicles include, but are not limited to, unilamellar vesicles and multilamellar lipid vesicles or liposomes. Such vesicles and liposomes may be made from a wide range of lipid or phospholipid compounds, such as phosphatidylcholine, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, glycolipids, gangliosides, *etc.* using standard techniques, such as those described in, *e.g.*, U.S. Patent No. 4,394,448. Such vesicles or liposomes may be used to administer compounds intracellularly and to deliver compounds to the target organs. Controlled release of a p97-composition of interest may also be achieved using encapsulation (*see, e.g.*, U.S. Patent No. 5,186,941).

Any route of administration which dilutes the composition into the blood stream, or at least outside of the blood-brain barrier, may be used. Preferably, the composition is administered peripherally, most preferably intravenously or by cardiac catheter. Intra-jugular and intra-carotid injections are also useful. Compositions may be administered locally or regionally, such as intra-peritoneally. In one aspect, compositions are administered with a suitable pharmaceutical diluent or carrier.

Dosages to be administered will depend on individual needs, on the desired effect, and on the chosen route of administration. Preferred dosages of p97 range from about 0.2 pmol/kg to about 2.5 nmol/kg, and particularly preferred dosages range from 2-250 pmol/kg; alternatively, preferred doses of p97 may be in the range of 0.02 to 2000 mg/kg. These dosages will be influenced by the number of compound moieties associated with each p97 molecule. Alternatively, dosages may be calculated based on the compound administered. Doses of p97-adriamycin comprising from 0.005 to 100 mg/kg of adriamycin are also useful *in vivo*. Particularly preferred is a dosage of p97-adriamycin comprising from 0.05 mg/kg to 20 mg/kg of adriamycin. Those skilled in the art can determine suitable doses for other compounds linked to p97 based on the recommended dosage used for the free form of the compound. p97 generally reduces the amount of drug needed to obtain the same effect. Additionally, p97 increases the maximum tolerated doses of these compounds because of the protective effect it has on the biodistribution. Those skilled in the art know how to select suitable dosages based on these and other considerations.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modification may be made thereto without departing from the spirit or scope of the appended claims.

IV. EXAMPLES

Example 1: Generation of a p97 expression system in BHK cells

Soluble P97 was obtained from a BHK TKneg (baby hamster kidney, thymidine kinase negative) (ATCC CRL 1632) cell line transfected with human p97

cDNA having a stop codon introduced at amino acid position 711 (glycine). The introduction of this stop codon resulted in the deletion of the GPI anchor attachment sequence. The cDNA was cloned into the expression vector pNUTΔH containing the DHFR gene allowing for selection with methotrexate. The structure of the preferred plasmid construct pNUTp97Δ-27 is illustrated in Figure 1. Transfection was performed using lipofectin, and selection was carried out in 0.5 mM methotrexate. Clones were screened for p97 production by FACS analysis and immunoprecipitation.

Example 2: Preparation and Purification of Human 97

Purified recombinant secreted human p97 was produced from transfected BHK cells. A BHK culture supernatant containing secreted p97 was first prepared (*see* part A) and p97 was then purified from the obtained BHK culture supernatant (*see* part B). Techniques used herein are described in Kemnard *et al.* (1993) *Biotech. Bioeng.* 42:480-86; and Food *et al.* (1994) *J. Biol. Chem.* 269:3034-40.

A. Production of BHK cell medium containing recombinant secreted human p97

Cell line: The BHK TKneg (baby hamster kidney, thymidine kinase negative) (ATCC CRL 1632) cell line transfected with human p97 as described in Example 1 and selected with 0.5 mM methotrexate was used. Clones were screened for p97 production by FACS analysis and immunoprecipitation.

Materials: The following materials were supplied by various commercial suppliers such as Gibco BRL, Faulding, *etc.* The BHK culture medium contained 1 M HEPES stock solution, 1 M Sodium azide stock solution, 100 mM Zinc sulphate stock solution, DMEM/Ham's F-12, Fetal Bovine Serum (FBS), N-[2-Hydroxyethyl] piperazine–N' [2-ethanesulphonic acid]) (HEPES), L-glutamine 100x, Zinc sulphate ($ZnSO_4.7H_2O$), Methotrexate (25 mg/ml), Phosphate buffered saline (PBS), Tryptan blue, Sodium azide, 0.05% Trypsin solution in 0.25 mM EDTA, EDTA. The following solutions were prepared: 500 ml of BHK culture media (DMEM/Ham's F-12 with additives); 100 ml of 1 M HEPES; 500 ml of 1 M sodium azide; 500 ml of 100 mM zinc sulphate.

Methods: Adherent cells frozen at -135°C at a density of $1x10^7$ cells/m were transfected. A 1 ml aliquot of frozen cells was rapidly thawed in warm water with shaking. 9 ml of culture medium were added to thawed cells in a 15 ml centrifuge tube drop by drop to reduce the effects of medium change. The cells were then allowed to stand for ten minutes at room temperature and then centrifuged at 1000 rpm (230xG) for

5 minutes at 4°C. The supernatant was carefully removed and the cell pellet was resuspended in 10 ml of culture medium and added to a 25 cm^2 T-flask. The cells were counted and the viability determined. The trypan blue exclusion method was used and the cells were counted using a haemocytometer. Incubation at 37°C in a 5% CO_2 humidified atmosphere was carried out until the cells became confluent. The supernatant was then removed and the cells washed by adding 25 ml of PBS. After pouring off the PBS, the cells were removed by adding 1 ml of a 0.05% trypsin solution in 0.25 mM EDTA and incubated at 37°C for 2 minutes in the CO_2 humidified incubator. The trypsin was immediately neutralized by adding 5 ml of culture medium. The cells were recovered from the T-flask surface by gently tapping the sides of the T-flask and pipetting the supernatant with a 10 ml pipette. The supernatant with the resuspended cells was recovered and placed in a sterile 15 ml polypropylene centrifuge tube and centrifuged at 1000 rpm (230xG) for 5 minutes at 4°C. After discarding the supernatant, the cells were resuspended in 10 ml of fresh culture medium. The cells were counted using the trypan blue exclusion method and a haemocytometer. The cell culture was then scaled up to a 175 cm^2 T-flask by adding 50 ml of fresh culture medium.

The number of cells to be added to the 50 ml of culture medium in order to obtain a seeding density of 1-2x10^5 cells/ml was determined. [For example, if the 10 ml cell suspension had 2x10^6 cells/ml then (50 x 2x10^5)/2x10^6=5 ml were added]. The cells were incubated until confluence at 37°C in a 5% CO_2 humidified atmosphere. Again, cells were counted using the trypan blue exclusion method and a hemocytometer. For the final scale up, a 1 l roller bottle was seeded with the cells from the 175 cm^2 T-flask. The 50 ml of supernatant were poured off and the cells were then removed by adding 10 ml of a 0.05% trypsin solution in 0.25 mM EDTA and incubating at 37°C for 2 min in the CO_2, humidified incubator. The trypsin was immediately neutralized by adding 50 ml of culture medium. The cells were recovered from the T-flask surface by gently tapping the sides of the T-flask and pipetting the supernatant with a 25 ml pipette. The supernatant with the resuspended cells was recovered and placed in a sterile 50 ml polypropylene centrifuge tube, centrifuged at 1000 rpm (230xG) for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in 25 ml of fresh culture medium. The cells were counted using the trypan blue exclusion method and a haemocytometer.

The cultured was scaled up to a 1 l roller bottle by adding 300 ml of fresh culture medium. The number of cells to be added to the 300 ml in order to obtain a seeding density of 1-2x10^5 cells/ml was determined. [For example, if the 25 ml cell

suspension had $1x10^7$ cells/ml, then $(300x\ 2x10^5)/1x10^7$=6 ml were added]. Incubation was carried out until confluence at 37°C in a 5% CO_2 humidified atmosphere. Cells were counted using the trypan blue exclusion method and a hemocytometer. The roller bottles were aerated daily with 5% CO_2 balance air and incubated at 37°C. The p97 secretion was monitored every two days using the Pandex assay method. All data was recorded in the worksheets. After approximately 7 to 10 days of culture, when the cells reached confluence, an additional 300 ml of culture medium were added. After a further 5 to 7 days of culture, the 600 ml of supernatant were recovered.

Since the cells were still viable and attached to the roller bottle, the culture may be re-fed with 300 ml of fresh culture medium, and topped up with a further 300 ml of culture medium after 3-5 days. The second 600 ml of supernatant were recover after a further 3-5 days of culture. Following this protocol, 1200 ml of supernatant with secreted p97 were recovered.

For recovering the p97 supernatant, the supernatant was centrifuged at 3000 rpm (2056xg) for 10 min at 4°C and the resulting supernatant was collected. The p97 concentration in the supernatant was determine (e.g., using a Pandex assay protocol). When necessary, the supernatant was concentrated 5 fold using a 30,000 MW cut-off ultrafiltration membrane. Preferably, the p97 concentration was >100 μg/ml. 20 mM sodium azide were added to the concentrated supernatant which was stored at 4°C until p97 purification.

For quality control determinations, once in the roller bottles, the BHK cultures were monitored every two days for p97 concentration. Typically, the concentration reached ~ 100 μg/ml. When this concentration was not achieved, the cell line was checked for mycoplasma contamination and the culture restarted from the first step. Cultures were checked for bacterial and yeast contaminations. If any contamination was detected, the culture was abandoned and restarted from the first step.

B. Procedures for the recovery and purification of the secreted p97 from the p97 transfected BHK culture supernatant

Reagents: 3 ml affinity columns were prepared with immobilized L235 on AffiGel 10 (*see*, Example 3, below); Elution buffer (0.1M citric acid, pH 2.5); Neutralization buffer (1M HEPES, pH 9.0); Column storage solution (PBS, 20 mM sodium azide); 1M Sodium azide stock solution; Citric acid ($C_6H_8O_7$); (N-[2-Hydroxyethyl] piperazine-N' [2-ethanesulphonic acid]) (HEPES); Sodium azide; Phosphate buffered saline (PBS). The following solutions were prepared: 500 ml of

buffer of citric acid at 0.1 M; 500 ml of 1M HEPES Neutralization buffer; 500 ml of storage solution (To 490 ml PBS add 10 ml of the stock 1 M sodium azide solution to give a 20 mM solution of azide in PBS); 500 ml of a 1 M stock azide solution.

Methods: The BHK culture supernatant containing secreted p97 prepared as described supra was purified. To purify approximately 100 ml of supernatant, a 3 ml of column of L235 immobilized on AffiGel 10 was used (*see* Example 3, below). The concentration of p97 in the solution to be purified was determined using a method such as a Pandex assay. The column storage solution was drained off under gravity, and the column was washed with 15 ml of PBS, by allowing the PBS to flow through the column under gravity. The sample was passed through the column at 15-18 ml/hr at room temperature and allowed to flow through under gravity. When necessary, the flow was adjusted using a drain valve attached to the column. The eluate was collected and saved for testing for p97 concentration determination using the Pandex assay method. (This was used to monitor the efficiency of the column). Following a wash with 15 ml of PBS (saved for p97 determination using the Pandex assay method), the buffer was allowed to flow through under gravity. Six 5 ml tubes were placed in a rack and labeled 1 to 6. p97 was eluted with 15 ml of Elution buffer and 3 ml fractions were collected in 5 ml tubes. The buffer was allowed to flow through the column under gravity and the fractions were neutralized with the neutralization buffer to pH 7.0±0.4. The pH was rapidly checked by testing 20 µl samples on pH strips in the range pH 5-10. Fractions were monitored by absorbance at 280 nm. The majority of p97 was eluted in fractions 2 and 3. These fractions were usually pooled and the p97 concentration determined using a method such as a Pandex assay method. The column was washed with 15 ml of PBS and stored in 10 ml column storage solution. Columns were stable for up to 1 year at 4°C.

For quality control tests, the purity of p97 was determined. The following standard assays were performed to characterize the p97 produced and to determine whether the produced p97 was at least 98% pure. Batches falling below the standard were discarded. Purity was determined by SDS-PAGE, Western blot, LC MS, or GC Mass Spectrometry. The concentration was determined by OD (extinction coefficient). Immunofluorescence assays (Pandex), and amino acid composition analysis were also performed. The identity was determined by Tryptic digest and MALDI-TOF MS and the reactivity by immunofluorescence assays

Determination of p97 concentration (~1 mg/ml) was carried out by OD, extinction coefficient ε1%@280=12 cm^{-1}, by Pandex assay and by amino acid composition.

Example 3: Production of an Anti-P97 Affinity Column

A method for preparation of an AffiGel column with L235 antibody for use in the purification of secreted recombinant p97 from BHK cell supernatant was designed. First, L235 anti-human p97 monoclonal antibodies were produced using the L235 hybridoma cell line. The L235 antibodies were then used to prepare an Affi-gel separation column. An alternative anti-p97 antibody HybC was also produced.

A. Production of L235 anti-human p97 monoclonal antibodies

Cell lines: The Hybridoma L235 -ATCC HB8446 L235 (M-19) cell line was used for producing the L235 antibodies. For the feeder layer, irradiated mouse embryonic fibroblast cells -ATCC X-56 were used.

The following items were supplied by standard commercial suppliers such as Gibco, EM Science, Sigma, BDH, *etc.*: RPMI; Hybridoma medium; 1 M Sodium azide stock solution; 1M HEPES stock solution; 50 mM β-mercaptoethanol stock solution; Fetal Bovine Serum (FBS); (N-[2-Hydroxyethyl] piperazine-N' [2-ethanesulphonic acid]) (HEPES); non-essential amino acids 100x; L-glutamine and Pen/Strep100x; L-proline 100x; β-mercaptoethanol; Phosphate buffered saline (PBS 10x); Trypan blue; Sodium azide. The following solutions were prepared: 100 ml of 1 M HEPES; 500 ml of 1 M sodium azide; 100 ml of 50 mM β-mercaptoethanol.

500 ml of hybridoma and feeder layer culture media were prepared and the pH was adjusted to 7.4±0.2. 500 ml of RPMI solution were prepared from the powder according to the manufacturer's instructions. The powder was emptied into 1 l beaker with a stirrer bar and 500 ml of DDH_2O were added and mixed at room temperature. If necessary, the pH was adjusted, using either 1 M hydrochloric acid or 1 M sodium hydroxide. In a 1 l glass beaker with a stirrer bar, at room temperature, 425 ml of freshly prepared RPMI were added as well as:

50 ml of FBS (heat inactivated at 57°C for 1 hr in a water bath)
10 ml of 1 M HEPES
5 ml nonessential amino acids 100x
5 ml L-glutamine 100x

5 ml L-proline 100x

0.5 ml 50 mM β-mercaptoethanol

After mixing at room temperature for ~ 10 min, the medium was sterile filtered through a 0.22 μm filter under vacuum in a laminar flow hood and stored in a sterile 500 ml media bottle at 4°C for up to 1 month.

The feeder cells were obtained from ATCC in polystyrene tubes with screw tops. The following steps were carried out in a laminar flow hood. A 1 ml aliquot of frozen cells was thawed rapidly in warm water with shaking. The thawed feeder layer cells were added to 50 ml of medium in a 50 ml polypropylene centrifuge tube and allowed to stand for ten minutes at room temperature. 2 ml of the cell suspension were added to 2x25 cm^2 T-flasks. 23 ml of the cell suspension were added to 2x150 cm^2 T-flasks. The cells were cultured for 1 day at 37°C in a 5% CO_2 humidified atmosphere. The medium was poured off into a glass beaker and replaced with fresh culture medium -- 10 ml in the 25 cm^2 T-flask, 50 ml in the 150 cm^2 T-flask. The cells were then cultured for another day at 37°C in a 5% CO_2 humidified atmosphere.

For the hybridoma culture, a 1 ml aliquot of frozen cells was thawed rapidly in warm water with shaking. 9 ml of medium were added to the thawed cells in a 15 ml centrifuge tube drop by drop to reduce the effects of medium change and the cells were allowed to stand for ten minutes at room temperature.

Following a centrifugation at 1000 rpm (230xg) for 5 minutes at 4°C, the supernatant was carefully discarded and the cell pellet resuspended in 10 ml of conditioned medium from the 175 cm^2 T-flask and added to a 25 cm^2 T-flask containing only the feeder layer. The cells were counted under the microscope using a haemocytometer and the viability determined using the trypan blue dye exclusion method. Following an incubation at 37°C in a 5% CO_2 humidified atmosphere until the cell density reaches 1x10^6 cells/ml and the viability >90%, viability was determined again using the trypan blue dye exclusion method and the cells were counted under the microscope using a haemocytometer. 10 ml of cells were transferred to the 175 cm^2 T-flask containing the feeder layer and 100 ml of culture medium. The 25 cm^2 T-flask culture was kept in order to reseed another 175 cm^2 T-flask culture. The approximate viable cell density of the cells was determined to be about 2x10^5 cells/ml, using the trypan blue dye exclusion method and counting the cells under the microscope using a haemocytometer. The cells in the 175 cm^2 T-flask culture were monitored until the

viability of the hybridomas fell below 60-70%. Again, the trypan blue dye exclusion method was used and the cells were counted under the microscope using a haemocytometer. The cell density and viability was ideally determined every 2 days. The antibody concentration was also measured every 2 days using the monoclonal assay. The supernatant containing cells was removed and centrifuged at 1000 rpm (230xg) for 10 min at 4°C and the cell free supernatant recovered (approximately $1x10^6$ cells/ml were left in the T-flask for the next culture --the feeder layer may be used for approximately 4 cell cultures) Add 20 mM sodium azide to the supernatant and store at 4°C prior to antibody purification.

For quality control tests, the culture were monitored every 2 days for cell viability and density, as well as the concentration of secreted monoclonal antibody. When the antibody concentration was not ~10 µg/ml when the cell density reached approximately $1x10^6$ cells/ml, the culture was abandoned and restarted. The cultures were checked for bacterial and yeast contaminations. If any contamination was detected, the culture was abandoned and restarted.

Further details on all procedures are described in the Antibody Handbook. Purity measurement were preferably performed using SDS-PAGE, IEF gel or LC. The concentration was typically determined using OD measurements or immunofluorescence assays (Pandex), and the affinity was evaluating by detecting p97 in Western blots or by using an ELISA titration method.

B. Preparation of an affinity column using L235 antibody (L235 immobilized AfiGel 10 column or L235 affinity column)

The purified L235 was provided in a buffer containing 0.1 M glycine HCl and 0.1 M Tris-HCl. The L235 was first transferred into a buffer containing 100 mM HEPES at pH 7.4±0.2 in a 15 ml Slide-A-Lyzer cassette with 3 changes of HEPES with ~24 hr between changes. L235 was concentrated to 15 mg/ml using 3 ml Centriprep or 15 ml Centricon concentrators (30,000 MW cut-off) according to the manufacturer's instructions.

To prepare a 3 ml L235 affinity column, 6 ml of AffiGel-10 (BioRad) suspension (~50/50 solution) were transferred to an empty 1 cm diameter glass column and drained. The column was washed with 15 ml of cold (4°C) dd H_2O, which were allowed to flow through the column under gravity. The bottom of the column was sealed with Parafilm and 3 ml of 15 mg/ml of L235 in 100 mM HEPES were added. The top of the column was sealed with Parafilm and the column was placed on a rocker at 4°C for 4

hours with gentle rocking so the antibody mixed well with the gel. The column was drained and the solution saved to check for efficiency of antibody binding. The concentration of any unbound antibody in the eluate was determined by the Pandex antibody assay method. The column was washed with 30 ml of PBS which were allowed to flow through the column under gravity. The column was stored at 4°C in 15 ml of column storage solution.

For quality control, the antibody binding efficiency was determined as follows. The OD of the L235 solution was measured at 280 nm before and after contact with the AffiGel. The % efficiency was determined using the following equation:

(Dilution x OD of 15 mg/ml of L235 x sample volume)-(Dilution x OD eluate x sample volume) x100

(Dilution x OD of 15 mg/ml of L235 x sample volume)

The efficiency was ideally >75%

C. Alternative method for purification of p97 using a HybC antibody affinity column

A HybC anti-human p97 monoclonal antibody was produced by culturing the HybC hybridoma cell line. This antibody was used as an alternative for L235 for the purification of p97 from BHK cell supernatants.

Cell line: Hybridoma C -33B6E4 produced by Dr. Shuen-Kuei Liao (Dept. Pathology and Pedriatrics, McMaster University, Hamilton Ont.)

For the hybridoma medium, 500 ml of DMEM solution were prepared from powder according to the manufacturer's instructions. The powder was emptied into a 1 l beaker with a stirrer bar and, after adding 500 ml of ddH_2O the solution was mixed at room temperature. The pH was checked to be ~ 7.4±0.2 and adjusted if necessary, using either 1M hydrochloric acid or 1M sodium hydroxide. In a 1 l beaker with a stirrer bar at room temperature, 430 ml of freshly prepared DMEM were added, as well as 50 ml FBS (heat inactivated at 57°C for 1 hr in a water bath), 10 ml of 1M HEPES, 5 ml L-Glutamine Pen/Strep, 5 ml non essential amino acids and 0.5 ml of 50 mM β-mercaptoethanol. The medium was mixed at room temperature for ~ 10 min, sterile filtered through a 0.22 µm filter under vacuum in a laminar flow hood and stored in a sterile 500 ml media bottle at 4°C for up to 1 month.

A 1 ml aliquot of frozen cells was rapidly thawed in warm water with shaking. 9 ml of medium were added to the thawed cells in a 15 ml centrifuge tube drop by drop to reduce the effects of medium change. The cells were allowed to stand for ten

minutes at room temperature and centrifuged at 1000 rpm (230Xg) for 5 minutes at 4°C. The supernatant was carefully removed and the cell pellet was resuspended in 10 ml of culture medium and added to a 25 cm^2 T-flask to count the cells and determine the viability using the trypan blue exclusion method and counting the cells using a haemocytometer. Following incubation at 37°C in a 5% CO_2 humidified atmosphere until the viable cell density reached $1x10^6$ cells/ml, the cell density and viability were determined as described above. The volume was scaled up to 50 ml by transferring the 10 ml contents of the 25 cm^2 T-flask to 75 cm^2 T-flasks and adding 40 ml of culture medium. The viable cell density was allowed to reach $1x10^6$ cells/ml. For the final scale up, 50 ml at $1x10^6$ cells/ml contents of the 75 cm^2 T-flask were used to inoculate 500 ml of media in a sterile 1 l spinner flask (inoculation viable cell density at ~$1-2x10^5$). The inoculation cell density was checked as described *supra*. The cells were cultured for approximately 10 to 15 days at 37°C in a 5% CO_2 humidified atmosphere until the cell viability fell below 80%. The cells density and viability was measured every 2 days, as described *supra*. The antibody concentration was also measured every 2 days using the monoclonal antibody assay and the data was recorded in the worksheets. The supernatant containing cells was removed and centrifuged at 1000 rpm (230xg) for 10 min at 4°C to pellet the cells. The cell free supernatant was carefully recovered and 20 mM sodium azide were added. The supernatant was stored at 4°C prior to antibody purification.

Quality control was tested as described *supra*.

Example 4: Preparation Of Apo And Holo P97

$FeCl_3$ or $^{55}FeCl_3$ may be used depending on the objectives of the study.

p97 (also called melanotransferrin; MTf) was concentrated by spinning 2 ml of purified MTf solution in a Centricon 30 tube for 12 min at 2000 X g. The filtrate was saved and the filter was washed with 100 µl of filtrate for 5 min at 500 X g. The concentration of the retentate was measured at 280 nm using the filtrate as blank. The molarity (moles/l) and concentration (mg/ml) were calculated with molar extinction coefficient (94420 abs l $mole^{-1}$) and (1.218 abs ml mg^{-1}). The concentrations and volume of the retentate were recorded.

A. Apo MTf

Fe and other metals were removed from MTf by dialysis as follows: 2 l of 0.1 M sodium acetate buffer pH 5.0, 0.001 M sodium citrate, 0.001 M EDTA were prepared. A Slide-A-Lyzer 10,000 MWCO dialysis cassette were hydrated in buffer for

30 s and the MTf retentate was introduced into the cassette and dialyzed in buffer for 3 hours. The buffer was changed to 2 l of 0.1 M NaCl, 0.020 $NaHCO_3$ and dialyzed for 1-2 hours. The dialysate was recovered and the volume recorded. The concentration was measured at 280 nm with dialysis buffer as blank and recorded. When necessary, the dialysate was concentrated.

B. Holo MTf

A buffer containing 5 mM $FeCl_3$ or $^{55}FeCl_3$ in 0.5 M HCl, 25 mM sodium citrate, and 1 M $NaHCO_3$ was prepared. The iron was chelated with citrate by adding 25 µl $FeCl_3$ or $^{55}FeCl_3$ to 50 µl sodium citrate, vortexing and waitng for 15 min. 20 µl $NaHCO_3$ were added and after 15 min the mixture was vortexed periodically. The CO_2 released from the solution was exhausted. 250 µl of MTf (2 mg/ml) were added, the mixture was vortexed and allowed to sit for 1 hour. 1 ml of 100 mM NaCl, 20 mM $NaHCO_3$ was added and introduced into hydrated Slide-A-Lyzer 10,000 MWCO. Dialysis was performed against 2 l of 100 mM NaCl, 20 mM $NaHCO_3$ for 1-2 h. The solution was recovered from the dialysis cassette, the volume recorded, and the concentration measured at 280 nm using dialysis buffer as blank. When necessary, the obtained solution was concentrated.

Example 5: Use of Chloramine T to Generate P97-compounds such as p97-Iodine

Materials and Methods: [125]Iodine was obtained from Amarsham Life Science Corp. Bovine serum albumin (BSA) was obtained from SIGMA. The human soluble apo-p97 was prepared according to example 2, above. Mouse apo-Tf was obtained from SIGMA (T-0523). Column PD-10 containing Sephadex" G-25 was obtained from Pharmacia Biotech.

Preparation of 125I-recombinant soluble p97 and holo-Tf:

Human recombinant soluble p97 was iodinated using a chloramine T procedure. The p97 (20µg/20µl prepared as above) was mixed with 20 µl of 0.5 M phosphate buffer and 1 mCi (1 Ci = 37 GBq) of $Na^{125}I$. Chloramine T was added, and 60 seconds later the reaction was stopped by adding 20 µl of sodium metabisulfate. Another 60 seconds later 100 µl of NaI was added and then eluted with 20 ml PBS containing 1% BSA. The radiolabeled proteins were separated from unreacted iodine using Sephadex G-25 gel filtration using PD-10 kit. The specific activities of ^{125}I-p97 and ^{125}I-Tf were 9-10 x 106 cpm/pmol, 9.13 x 10^6 cpm/pmol respectively. The trichloroacetic acid (TCA – 10%) precipitability was >90%, and generally >99%.

In several experiments p97 was iron saturated according to the method of Larrick and Creswell.

Example 6: Preparation of P97-gold

Human p97 was conjugated with 5 nm colloid gold by a standard gold linking protocol (performed at British BioCell International). Briefly, 5nm gold particle sols were prepared in chloroauric acid solution plus K_2CO_3. White phosphorus in diethylether was then added. The mixture was reacted at room temperature for 15 min, then boiled and refluxed until the color of the suspension turned from brownish to red. The solution was then cooled and used. p97 with 5nm gold colloid was produced at a high quality, a high percentage of singlets (99%) with no clusters of larger than 3 particles observed. The concentration of human p97 was 70 μg/ml. The coefficient of variation of particle size was 11%. Bovin Serum Albumin (BSA) was conjugated to 5 nm colloid Gold by BBI and tested. 96% were singlets with no clusters larger than triplets. The concentration of BSA was 20 μg/ml and the coefficient of variation of particle size was 11.6%.

For preparing the p97Ab-gold, mouse anti-human p97 mAb (L235) was conjugated with 5 nm gold as described above. The concentration of mAb employed was 95 μg/ml.

Example 7: Cross-linking of p97 to Compounds Using SATA Derivatives

Methods of linking p97 to a compound using SATA derivatives (Pierce) are provided herein and may be used to make a wide variety of p97-compounds.

A. p97-Doxorubicin

For preparing the SATA derivatives, p97 was used at 1.3 mg/ml. 10 mg of SATA were dissolved in 1.0 ml DMSO, immediately before use. 1 ml of p97 was combined with 100 μl of SATA (10 mg/ml) in an eppendorf tube, and allowed to react at room temperature for 60 min.

For the purification, Excellulose GF-5 desalting columns were used for separation. The columns were equilibrated with 10 ml of Buffer 1. 1.1 ml of reaction mixture were applied to the column. The fractions were collected at 1 minute intervals and monitored at A280. The fractions containing p97 were pooled.

For the deacetylation reaction, the deacetylation solution contained 0.35 g of Hydroxylamine-HCl and 0.073 g of EDTA in 8 ml of 62.5 mM Sodium Phosphate, pH

7.5 buffer. The pH was readjusted to 7.5 with NaOH and the final volume was brought up to 10 ml. The final concentration of this buffer was 50mM Sodium Phosphate, 25 mM EDTA, 0.5 M Hydroxylamine, pH 7.5. 0.4 ml of the pooled p97 fractions were combined with 40 µl of deacetylation solution in an eppendorf tube and allowed to react for 2 hours at room temperature.

A solution of sulfo-SMCC in DMSO at 33 mM was used. Doxorubicin was used at 2.5 mg/ml (4mM) in 25% PBS and 75% DMSO at pH 7.2. 60 µl of Doxorubicin were combined with 60 µl of prepared Sulfo-SMCC and incubated for 3.5 hours at room temperature.

For conjugation, 0.440 ml of deacetylated p97 were mixed with 120 µl of a doxorubicin-SMCC mixture and incubated at 4°C overnight.

The purification steps described above were repeated. The columns were equilibrated with 10 ml of PBS. Characterization of the resulting product by mass spectrometry and colorimetric assays revealed that from 0.1 to 20 molecules of Adriamycin (Adr) were conjugated per molecule of p97, with an average of 0.5-5 molecules of Adr. Specific p97:Adr ratios were obtained by optimizing the reaction conditions. In some applications, separation on a p97:adr molar ratio basis of the products generated in this linking reaction was desired (*i.e.*, separating p97:adr 1:1 from p97:adr 1:10). Purifying and separating these products was achieved by standard methods in the art, such as HPLC on an AKTA purifier (Pharmacia).

In some experiments p97-adr was further labeled with I-125, by using the p97-Adr generated above in a simple chloramine T reaction as described *supra*.

B. p97-Cisplatinum

Conjugation of cisplatinum to p97 was carried out as in Example 6A, with the replacement of doxorubicin by the same concentration of cisplatinum, and changing the SMCC concentration to 84 mM.

C. p97-Horse Radish Peroxidase

Conjugation of horse radish peroxidase to p97 was carried out as in Example 7A with the replacement of doxorubicin with 10 mg/ml purified horse radish peroxidase and changing SMCC concentration to 10 mM. Additionally, the p97 concentrations were preferably up to 4.5 mg/ml.

D. p97-cisplatinum-adriamycin

In a different experiment, a multiple combination of p97-compound was made using the SATA protocol of Example 7A with the following modifications. For p97-cisplatinum-adriamycin the reaction required the following mol:mol ratios:

CisPt: ADR = 1:1

ADR: SATA= 1:2

CisPt:SMCC = 1:10

CisPt:p97 (at start) = 45:1

The solutions were initially prepared in standard buffers as follows: CisPt = 2.5 mg/ml; ADR = 2.5 mg/ml; SATA = 5.25 mg/ml for ADR; SATA = 10 mg/ml for p97; SulfoSMCC = 36.5 mg/ml (84 μM); p97 = 1.3 mg/ml.

For generating CisPt-ADR complex, 4 x 75 μl CisPt and 4 x 75 μl SMCC were first incubated for 3.5 hours at room temperature. Separately, 4 x 145 μl ADR + 4 x 55 μl SATA were incubated for 1.5 h at room temperature and 4 x 200 μl ADR-SATA + 4 x 50 μl deacylation solution were incubated for 2 h at room temp. Finally, 4 x 250 μl dADR-SATA + 4 x 150 μl CisPt.-SMCC were incubated for 1-3 h at room temp (Compound A).

For generating the p97-SATA derivative, 8 x 500 μl p97 + 8 x 50 μl SATA were incubated for 1 h at room temperature, desalted over 4 desalting columns. Fractions 6-11 were collected (1' increments). 16 x 200 μl p97-SATA + 16 x 20 μl deacylation solution were then incubated for 3 h at RT (Compound B).

For generating the p97-CisPt-Adriamycin complex, 16 x 220 μl dp97-SATA (Compound B) + 16 x 100 μl CisPt-ADR (compound A) were incubated overnight at 4C and desalted over 4 columns.

E. Methods of influencing linkage ratios

Different mol:mol ratios of a p97-compound may be obtained. For some applications a 1:1 ratio may be satisfactory, for others, 1:10 or higher. For instance, it has been found that increasing the ratio of the activated compound or therapeutic agent (*e.g.*, ADR-SMCC) to the activated p97 (*e.g.*, p97-SATA) results in an increased Molecular Substitution Ratio (MSR) (Figure 1). Thus, the p97–compound ratios are tunable according to the needs and desires of the particular usage using the methods of the present invention.

In addition to adjusting the ratios of the compound or therapeutic agent and p9&, another method for improving the linkage ratios involves purifying the ADR-SMCC conjugate before linking it to p97-SATA. This additional step removed contaminants which blocked free amino groups on the p97.

Example 8: Preparation of a p97-compounds, such as p97-Alexa by direct linkage

A particularly preferred method of making a p97-compound does not require SATA/SMCC activation of p97 and the compound, but allows direct conjugation of the compound to p97. Such a method is provided by NHS ester (anhydroxy succinimidyl) linkage directly through the available amines on p97.

This method was used for generating p97-Alexa. This composition was made using the dye Alexa Fluor 546 carboxylic acid, succinimidyl ester, sodium salt (MW ~ 1079) in a standard bicarbonate/hydroxylamine reaction with p97 according to the Alexa Fluor™ protein labelling kit (Molecular Probes, Inc. kit # A-10237).

Example 9: Delivery and treatment of non-CNS organs with p97-compounds

p97 or BSA (bovine serum albumin, control) were prepared and iodinated with I^{125} using the chloramine T protocol, above. Where indicated, p97- I^{125} was treated according to the methods described above to generate Apo p97- I^{125} (essentially iron free p97) and Holo p97- I^{125} (p97 loaded with $FeCl_3$). $1x10^7$ DPM of sample was prepared in 200 µl buffer (100 mM NaCl and 20 mM HCO_3) and administered to C57 black mice (16-20 g) by tail vein injection. At the indicated time point, mice were given an overdose of Ketamine/Xylazine anaesthetic mix. The chest was opened and blood was removed with a 27 gauge needle via cardiac puncture. The left atria was snipped open and the mouse was perfused with heparinized saline to flush out any serum associated counts from the vascular system. Organs were then removed using standard techniques. I^{125}

counts were read directly from whole organs in a gamma scintillation counter. The tissue/serum ratio at 60 minutes after injection of Apo P97-I125, Holo p97-I125 and BSA-I125 were recorded (Figure 2). Every organ demonstrated significantly increased uptake of p97 compared to BSA. No significant difference was identified between the Apo and Holo forms of the protein (kidney results not confirmed as significant). These results identified that the non-CNS p97 target organs heart, lung, liver spleen and kidney had a preferential uptake of p97 over BSA. At 1 hour, BSA linked compounds remained in serum to a significantly higher degree than p97 linked compounds. The relative increase at 15 minutes in p97 uptake by non-CNS p97 target organs over BSA is illustrated in Figure 3. These results indicated that compounds such as therapeutic agents were preferentially targeted to these organs by linking them to p97. Again no difference between Apo and Holo forms of p97 were observed. The relative increase in p97 uptake by the non-CNS p97 target organs was maintained over at least 60 minutes (Figure 4). Brain tissue demonstrated a significantly greater relative increase of p97 uptake over BSA uptake.

Example 10: Linking of compound to p97 significantly influences biodistribution of compound.

p97 was conjugated to [^{14}C]ADR to generate p97-[^{14}C]ADR using the techniques described above. The generated p97-[^{14}C]ADR was found to have a specific activity of 57mCi/mmole. A solution of 500000 dpm/mouse of this formulation in 100 µl was injected intraperitoneally into each mouse. The same amount of free [^{14}C]ADR was injected into comparative mice. At 1 hour after injection, mice were terminated and organs were prepared as described above. Tissue was solubilized and read in a scintillation counter.

The accumulation at various organs of p97-ADR was recorded (Figure 5). The data demonstrated that ADR linked to p97 had a significantly different biodistribution than free ADR. The results demonstrated that p97 enhanced the delivery of ADR to the spleen; and allowed a longer serum circulation time for the drug than free ADR. Additionally, p97 exerted a strong protective effect on the heart, liver, and kidney and reduced the accumulation of ADR at those organs.

A significant difference in tissue to serum ratio of heart tissue between p97-ADR and ADR was observed (Figure 6). These results demonstrated that p97 exerted a major protective effect on cardiac tissue. Linkage of the cardiotoxic drug ADR

to p97 significantly reduced cardiotoxicity of the dose of drug. This permits the administration of a much higher amount of ADR than previously possible, without increasing the cardiotoxic consequences of such treatment.

Example 11: Ability of p97-Adriamycin to inhibit growth of a subcutaneous tumour in a standard mouse model

The C6 rat glioma (American Type Culture Collection CRL-2199) which expresses the lacZ reporter gene product and produces E-coli derived beta galactasidase activity was used for all the *in vivo* studies. The mice used for the subcutaneous tumor implantation were 7-9 week old athymic NSWNU(m) Swiss nu/nu. All mice were housed in micro isolated cages (5 mice per cage) under positive air pressure in a Hepa filtered ventilated animal rack. The experimental protocol was approved by the University of British Columbia committee on animal care (Protocol number A99-0198).

The C6 cells maintained on culture plates were suspended by the addition of trypsin and then counted, washed and suspended in sterile PBS to obtain a solution containing the appropriate number of cells for injection. For example, in the initial trial a solution of $1x10^6$ cells/ml was used, and $1x10^5$ cells were delivered per 100 µl injection. In some cases, the volume was reduced to 50 µl. The site of subcutaneous injection was on the right flank of the animal, approximately 1 cm below and 1 cm posterior to the shoulder. Sterile procedures were used including wiping the injection site before and after with alcohol swabs to prevent infection. Subcutaneous C6LacZ, $1x10^6$ injection resulted in visible tumors at around 1 week in nu/nu mice. Growth was then rapid over the next two weeks with tumor sizes reaching up to 10% of body weight (*i.e.*, termination of protocol).

P97-Adr was prepared according to the protocols described above. The formulation administered was characterized by spectrophotometric analysis at 280 and 486 nm to give a reading of the protein at 280 (p97) and of the ADR absorbance at 486. The ratio of p97:ADR was 1:10.

All treatments were by tail vein administration in 200 µl volumes. 6 mice were used in each of the treatment groups. In group A, PBS was administered to mice on days 8, 12, and 15. In group B, Adriamycin (5 mg/Kg body weight of Adriamycin) was administered to mice on days 8, 12, and 15 (Total ADR = 15 mg/kg). Mice from group C were administered P97-Adr (0.05 mg/Kg body weight of Adr.) on days 8, 12, & 15. (Total ADR = 0.15 mg/kg).

Tumor growth was measured using digital calipers to record length, width, and height. Tumor volume was calculated using the formula: Volume = $(\pi/6)$ x (length x width x height) or Volume = (length^2 /2) x width for a method that avoided relying on the harder to measure value of tumor depth or height. Studies were terminated when the tumor reached 10% of body weight.

Tumor growth was heterogeneous with some animals bearing small tumors and some large. A reduction in C6 glioma subcutaneous tumor growth was obtained with both free Adr and p97-Adr (Figure 7). The difference between the ADR and p97-ADR treatments were not statistically significant. However, the dosages of Adr in the p97-Adr formulation were 100 fold lower than in the free Adr, thus demonstrating a major increase in activity when linked to p97. A more detailed illustration of day 21 data is showed in Figure 8.

Example 12: Utility of p97-Adr administered by intraperitoneal injection to treat a subcutaneous C6 Glioma tumour

C6 glioma mouse models were prepared and used as described above. Treatment was modified as follows. Intraperitoneal injection was used to avoid delivery problems with necrosis and inflammation of the tail veins since intraperitoneal injection seemed to deliver just as much ADR and p97-ADR to serum and tissues. The dosage of ADR in the p97-ADR was equivalent to 1/20th the amount of free ADR delivered. 3 groups of mice (n = 9) were used in each test group. On Day 0, all received a subcutaneous injection of C6 cells ($1x10^6$) in 100 µl of PBS. 3 different treatments were started. In treatment A, 100 µl of PBS were given intra-peritoneally (I.P). In treatment B, mice were received 100 µl of ADR (20 mg/Kg) I.P. In treatment C, 100 µl of p97.ADR (delivering 1mg/Kg ADR) were given I.P to the mice. Treatments were administered on day 2, 5, 7 and 10. Body weights and tumor dimensions were recorded every two or three days.

Consistent with the above results, i.p. injection of p97-ADR induced a significant inhibition of tumor growth by day 21 after injection of tumor cells (Figure 9). Free ADR also resulted in tumor growth inhibition, though relative rates of tumor inhibition are not comparable, because the dose of free ADR was 20 times higher (20 mg/kg body weight) than the ADR in the p97-ADR formulation (1 mg/kg body weight).

Example 13: Uptake of p97-gold by non-CNS target organs

These experiments showed that p97-gold was endocytosed by specific uptake vesicles upon delivery to the liver.

BSA-5 nm gold (20 μg/ml gold)(100 μl) and p97-5 nm gold (70 μg/ml gold)(100 μl) were injected into the jugular vein of young adult mice (C57Bl/6) and allowed to circulate for approximately 30 min. Subsequently, the animals were perfused with fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4) via the abdominal aorta. Organs were removed, post-fixed and rinsed in buffer. Each organ was then sliced cross-sectionally (30-50 μm) using a vibratome and collected in buffer before further processing. Vibratome sections from animals injected with p97-5 nm gold conjugate were buffer rinsed, post-fixed in osmium tetroxide. Subsequent to this procedure, the tissue sections were processed through into resin using standard protocols for EM (dehydration through alcohols, infiltration with Epon resin, polymerization in oven), and thin sections were cut and examined with an electron microscope.

A composite electromicrograph identifying sites of specific p97-gold uptake in liver tissue is shown in Figure 10. Arrows identify gold particles. Gold was identified in specific uptake vesicles in a variety of cell types in the liver tissue. Similar results were obtained from other non-CNS p97 target organs such as the lung.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

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The disclosures of all articles and references, including patents, patent applications and publications, are incorporated herein by reference for all purposes.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(ii) TITLE OF INVENTION:

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE:

(B) STREET:

(C) CITY:

(D) STATE:

(E) COUNTRY:

(F) ZIP:

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Patent In Release #1.0, Version #1.25

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME:

(B) REGISTRATION NUMBER:

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(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE:

(B) TELEFAX:

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
GCGGACTTCC TCGG 14

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
TCGCGAGCTT CCT 13

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
CTCAGAGGGC CGCTGCGCCC 20

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCAGCGCAGC TAGCGGGGCA G 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACACCAGCGC AGCTCGAGGG GCAGCCG 27

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGCTACGTA CTCGAGGCCC CAGCCAGCCC CGACGGCGCC 40

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCGTACGTA TGATCATCAG CCCGAGCACT GCTGAGACGA C 41

WHAT IS CLAIMED IS:

1. A pharmaceutical composition, said pharmaceutical composition comprising:

(a) a compound conjugated to p97 or to a fragment thereof; and

(b) a pharmaceutically acceptable carrier.

2. A pharmaceutical composition, said pharmaceutical composition comprising:

(a) a compound conjugated to p97 or to a fragment thereof; and

(b) a pharmaceutically acceptable carrier,

wherein said pharmaceutical composition targets said compound to a non-CNS p97 target organ.

3. The composition of claim 2, wherein said compound is selected from the group consisting of therapeutic agents, prophylactic agents and diagnostic agents.

4. The composition of claim 2, wherein said compound is selected from the group consisting of chemotherapeutic agents, antibiotics, antiinfective agents, anti-fungal agents, nucleic acids, peptide hormones, base elements, metals, enzymes, and radionuclides.

5. The composition of claim 2, wherein said compound is selected from the group consisting of adriamycin, doxorubicin, daunorubicin, cisplatin, taxol, taxotere, amphotericin B, insulin, somatostatin, angiostatin, endostatin, morphine and therapeutic base elements.

6. The composition of claim 2, wherein said compound comprises at least two different compounds.

7. The composition of claim 2, wherein said compound conjugated to p97 or to a fragment thereof does not have neurological activity at a clinically recommended dose.

8. The composition of claim 2, wherein said compound is selected from the group consisting of cardiotoxic agents.

9. The composition of claim 2, wherein said conjugation of said compound to p97 or to a fragment thereof comprises an organic moiety containing an alkyl, an aryl or an amino acid backbone.

10. The composition of claim 9, wherein said organic moiety contains an amide, ether, ester, hydrazone, or disulphide linkage or any combination thereof.

11. The composition of claim 9, wherein said conjugation is stable at physiological pH, but unstable at intracellular pH.

12. The composition of claim 9, wherein said organic moiety is sensitive to cleavage by enzymes localized at p97 target organs.

13. An intermediate in the preparation of a p97-compound, said intermediate comprising p97 or a fragment thereof, wherein said p97 or said fragment thereof comprises a free amine group or a free thiol group.

14. The composition of claim 2, wherein said conjugation is by a sulfhydryl linker.

15. The composition of claim 2, wherein the molar ratio of said compound to said p97 or to said fragment thereof is at least 2:1.

16. The composition of claim 2, wherein the molar ratio of said compound to said p97 or to said fragment thereof is at least 5:1.

17. The composition of claim 2, wherein the molar ratio of said compound to said p97 or to said fragment thereof is at least 10:1.

18. The pharmaceutical composition of claim 2, wherein said composition comprises a dosage unit of from about 0.02 to about 2000 mg/kg of said p97 in the form of p97-compound.

19. The dosage unit of claim 18, wherein said compound is adriamycin, cisplatin or an analog thereof.

20. The dosage unit of claim 18, wherein said dosage unit contains from about 0.1 to about 100 mg/kg of said compound.

21. The dosage unit of claim 18, wherein said dosage unit comprises a dosage of from about 0.1 to about 10 mg/kg of p97.

22. Use of a p97-compound, wherein said compound is selected from the group consisting of adriamycin, doxorubicin, daunorubicin, cisplatin, taxol, taxotere, amphotericin B, insulin, somatostatin, angiostatin, endostatin, morphine and therapeutic base elements, in the manufacture of a medicament for targeting the compound to a non-CNS p97 target organ.

23. Use of a p97-compound, wherein said compound is selected from the group consisting of adriamycin, doxorubicin, daunorubicin, cisplatin, taxol, taxotere, amphotericin B, insulin, somatostatin, angiostatin, endostatin, morphine and therapeutic base elements, in the manufacture of a medicament for treating a disorder at a non-CNS p97 target organ.

24. Use of a p97-compound in the manufacture of a medicament for treatment of a disease associated with the liver, lung, kidney or spleen.

25. The use of a p97-compound in the manufacture of a medicament having reduced cardiotoxicity.

26. The use of a p97-compound in the manufacture of a medicament for a therapeutic use where cardiotoxicity is a dose limiting condition of the compound when the compound is not linked to p97.

27. A method for increasing the relative delivery of a compound to a non-CNS p97 target organ, said method comprising the steps of:

(a) conjugating a compound to p97 or to a fragment thereof to generate a p97-compound;

(b) administering said p97-compound to an animal having a disorder associated with a non-CNS p97 target organ, wherein the amount of compound delivered as part of the p97-compound to said organ is increased relative to delivery of said compound when said compound is not conjugated to p97 or to a fragment thereof and administered at an equivalent dose.

28. A method for targeting a compound to a non-CNS p97 target organ, said method comprising the steps of:

(a) conjugating a compound to p97 or to a fragment thereof to generate a p97-compound;

(b) administering the p97-compound to a patient having a disorder associated with a non-CNS p97 target organ, wherein the patient experiences increased delivery of the compound to said organ compared to when said compound is not conjugated to p97 or to a fragment thereof and is administered at an equivalent dose.

29. A method for reducing the cardiotoxicity of a cardiotoxic compound, the method comprising the steps of:

(a) conjugating a cardiotoxic compound to p97 or to a fragment thereof to generate a p97-compound;

(b) administering said p97-compound to a patient in need thereof, wherein said p97-compound is less cardiotoxic then the compound when it is not conjugated to p97 or to a fragment thereof and administered at an equivalent dose.

30. A method for increasing the therapeutic index of a cardiotoxic compound, said method comprising the steps of:

(a) conjugating the cardiotoxic compound to p97 or to a fragment thereof to generate a p97-compound;

(b) administering said p97-compound to a patient at a dosage that would result in unacceptable cardiotoxicity when the compound is not conjugated to p97 or to a fragment thereof and is administered at an equivalent dose.

31. The method of claim 30, wherein said compound is adriamycin and said dosage of adriamycin in the p97-compound is greater than the recommended dose of the compound in the free form.

32. A method of treating a disease in a non-CNS p97 target organ, said method comprising administering to a subject in need thereof a pharmaceutically effective amount of a p97-compound.

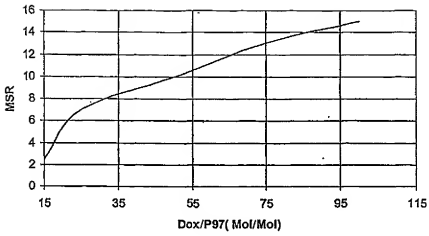

FIGURE 1

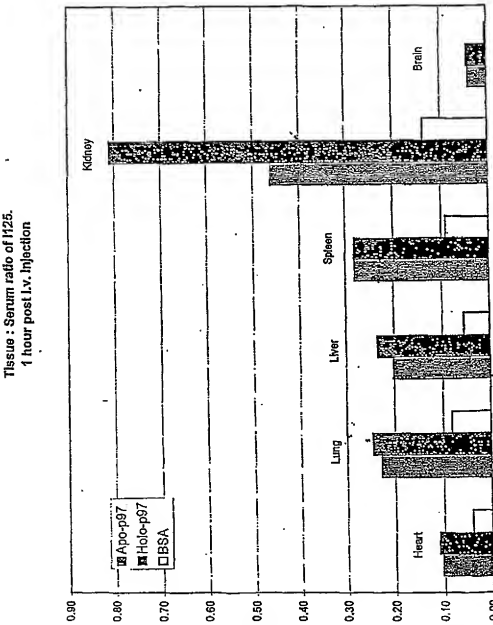
Tissue : Serum ratio of I125.
1 hour post I.v. Injection
Tissue / Serum ratio
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Apo-p97
Holo-p97
BSA
Kidney
Spleen
Liver
Lung
Heart
Brain

FIGURE 2

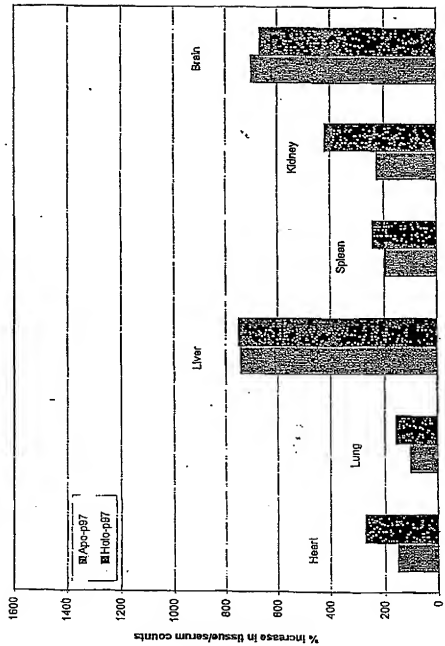
Apo & Holo I125-p97 15 mins:
% increase in uptake over I125-BSA
% increase in tissue/serum counts
1600
1400
1200
1000
800
600
400
200
0
Apo-p97
Holo-p97
Heart
Lung
Liver
Spleen
Kidney
Brain

FIGURE 3

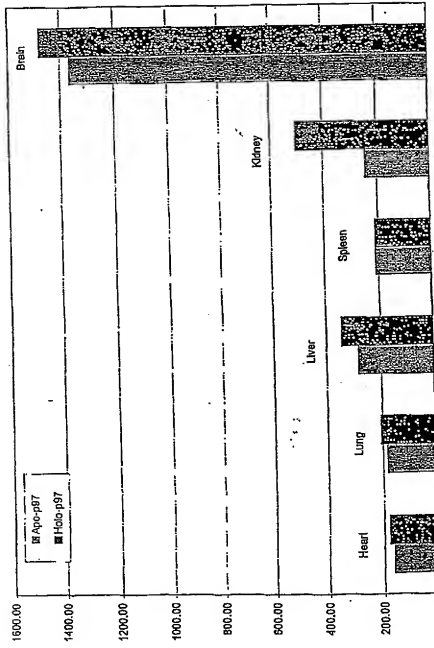
Apo & Holo I125-p97 60mins:
% Increase in uptake over I125-BSA
Apo-p97
Holo-p97
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
Heart
Lung
Liver
Spleen
Kidney
Brain

FIGURE 4A

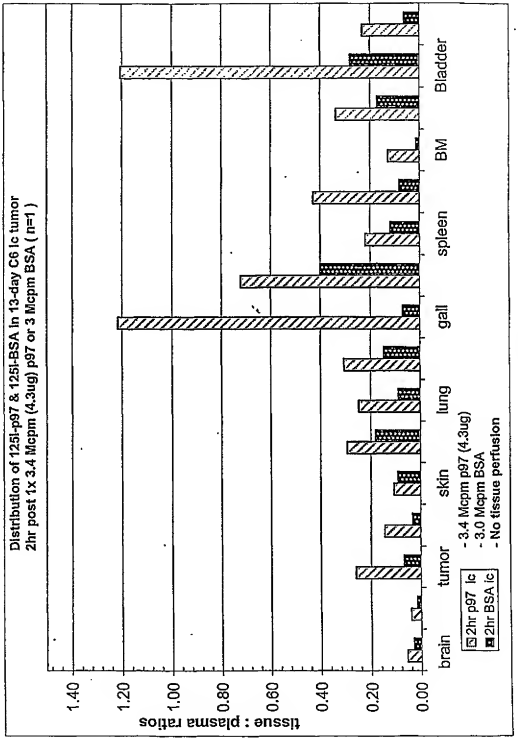
Distribution of 125i-p97 & 125i-BSA in 13-day C6 lc tumor
2hr post 1x 3.4 Mcpm (4.3ug) p97 or 3 Mcpm BSA (n=1)
tissue : plasma ratios
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
brain
tumor
skin
lung
gall
spleen
BM
Bladder
2hr p97 lc
2hr BSA lc
- 3.4 Mcpm p97 (4.3ug)
- 3.0 Mcpm BSA
- No tissue perfusion

FIGURE 4B

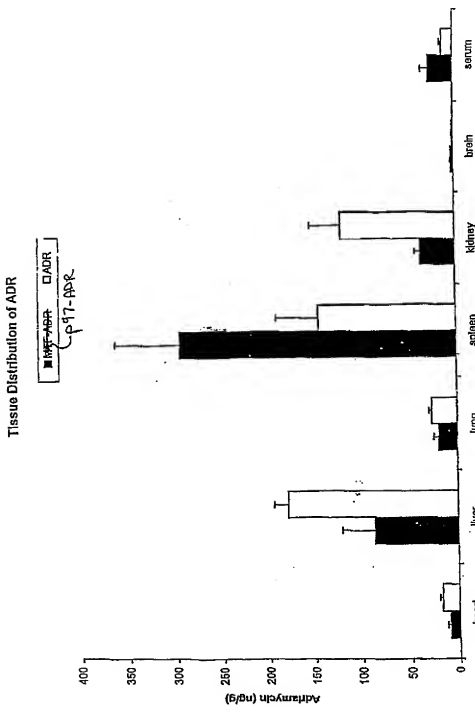
Tissue Distribution of ADR
MS-ADR
DADR
P17-PAR
Adriamycin (ng/g)
400
350
300
250
200
150
100
50
0
heart
liver
lung
spleen
kidney
brain
serum
Tissue

FIGURE 5

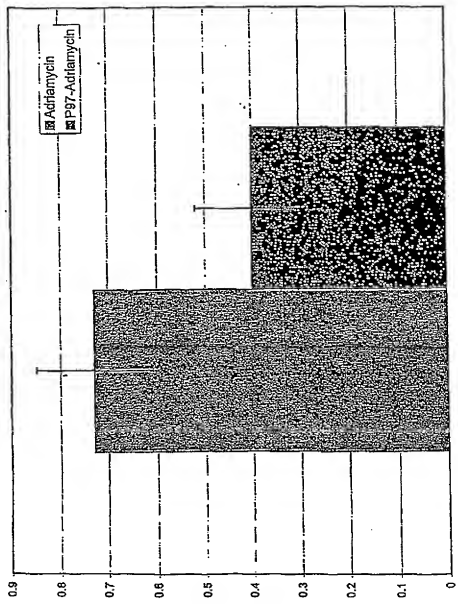
C14ADR and C14ADR-p97 uptake in heart tissue
Adriamycin
P97-Adriamycin
Tissue to serum ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

FIGURE 6

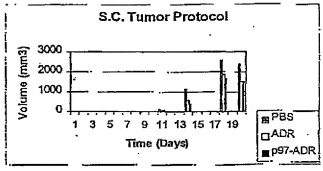

Figure 7: Effect of ADR and p97-ADR by i.v. administration on tumor inhibition

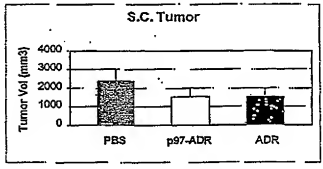

Figure 8: Effect of ADR and p97-ADR by i.v. administration on tumor inhibition at day 21.

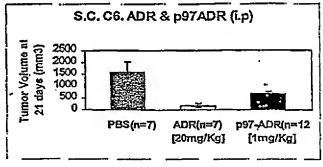

Figure 9: Subcutaneous C6 glioma tumor growth inhibition by p97-ADR administered intraperitoneally.

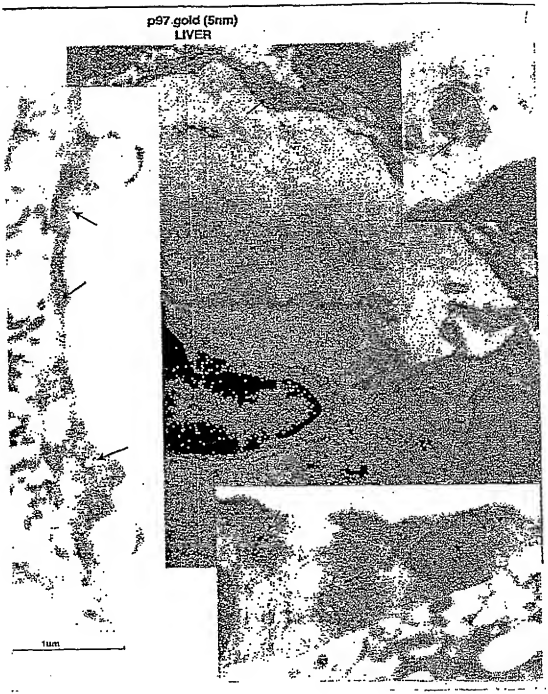

FIGURE 10

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(57) Abstract

A process is provided for coupling glycols to macromolecules through glycosylations on those macromolecules, rather than through amino or carboxyl groups on the macromolecule backbone. This produces macromolecules having decreased immunogenic response, and maintained activity. The present process for glycolation of a glycosylated macromolecule comprises activating a polyalkylene glycol; reacting the activated polyalkylene glycol with a diamino compound, whereby the activated polyalkylene glycol is coupled to the diamino compound through one of its amino groups; oxidizing the macromolecule to activate at least one glycosyl group therein; and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule. The result is a glycolated glycosylated macromolecule, wherein a glycol is bonded to the macromolecule through its glycosylations.

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GLYCOLATION OF GLYCOSYLATED MACROMOLECULES

Background of the Invention

The use of polypeptides in circulatory systems for the purpose of engendering a particular physiological response is well known in the medicinal arts. Among the best known polypeptides utilized for this purpose is insulin, which is used in the treatment of diabetes. Another group of polypeptides to which great therapeutic potential has been attributed are various enzymes. A principal factor which has severely limited the use in therapeutics of polypeptides is that most of these compounds elicit an immunogenic response in body fluids, evidenced by changes in the composition of the circulatory system, i.e., the production of antibodies to the polypeptides. This effect has one or both of two secondary consequences: first, neutralization of the polypeptides by the antibodies thus produced; second, and more seriously, the development of an allergic response.

Neutralization of polypeptides by antibodies is believed to be responsible for the rather low residence time of insulin in the human circulatory system; hence, persons afflicted with diabetes are forced to inject themselves fairly frequently with fresh doses of insulin. In the case of parenterally administered enzymes, not only is there the problem of neutralization of the polypeptide and the subsequent negation of its physiological activity, but also the extremely undesirable elicitation of an allergic reaction.

Overall, a limitation to the potential therapeutic benefit derived from the clinical use of polypeptides is

their potential for eliciting such immune response in the circulatory system. This immune response may be caused by aggregates in the material prior to injection as described by R. Illig (1970), J. Clin. Endocr., 31, 679-688, W. Moore (178), J. Clin. Endrocrinol. Metab., 51, 691-697. The antibody production may decrease or eliminate the desired biological function of the polypeptide, sometimes by causing reduced residence time in the circulatory system (reduced half-life) or by modifying the molecule by virtue of the antibody-polypeptide interaction.

The problems set forth hereinabove are well recognized, and various approaches have been taken in attempts to solve them. The attachment of enzymes to insoluble supports has been the subject of a great deal of work. Reviews dealing with this subject will be found in Silman and Katchalski, Ann. Rev. Biochem., 35, 387 (1966), and Goldstein, Fermentation Advances, Academic Press, New York (1969), page 391. This approach, however, while of academic interest, does not provide injectable long-life polypeptides. Another approach which has been taken to provide polypeptides of lengthened in vivo life has been the microencapsulation of enzymes which has been discussed in numerous articles by Chang and coworkers, namely, Science, 146, 524 (1964); Trans. Am. Soc., 12, 13 (1966); Nature, 218, 243 (1968); Can. J. Physiol. Pharmacol., 45, 705 (1967). A further approach has been the heat stabilization of enzymes by attaching carboxy methylcellulose to an enzyme such as Trypsin (Mitz and Summaria, Nature, 198, 576 (1961), and the attachment of proteases to hydrophilic carriers (Brummer et al., Eur. J. Biochem., 25, 129 (1972). These approaches, however, do not provide polypeptides in a soluble form, which form is the most desirable for injection and dosage control of injectable materials. Yet a further approach has been the attachment of synthetic polymers to polypeptidal proteins. A review of this work is found in Sela, "Advances in Immunology," 5, 30 (1966), Academic Press,

New York. In this work, it has been shown that while homopolymers of amino acids are nearly all non-immunogenic, when these polymers are attached to immunogenic proteins, the immunogenic activity is not masked, and antibodies are produced in test circulatory systems. For example, while polyglycine itself is non-immunogenic, when attached to a protein, that conjugated protein becomes a hapten. Similarly, while dextran itself is slightly immunogenic, when coupled to insulin, the insulin-dextran-coupled material is believed to become substantially immunogenic.

Other modifications of polypeptides include the modification of proteins with substantially straight-chain polymers such as polyethylene (PEG) or polypropylene glycol (PPG).

For example, U.S. Patent No. 4,055,635 discloses pharmaceutical compositions comprising a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as polysaccharides.

U.S. Patent No. 4,088,538 discloses a reversibly soluble, enzymatically active polymer enzyme product comprising an enzyme covalently bonded to an organic polymer such as polyethylene glycol.

U.S. Patent No. 4,415,665 discloses a method of conjugating an organic ligand containing at least one primary or secondary amino group, at least one thiol group, and/or at least one aromatic hydroxy group (described in column 3, lines 19-36) to a polymeric carrier with at least one hydroxyl group (described in column 2, lines 42-66).

U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1-proteinase inhibitor with a polymer such as PEG or methoxypolyethylene glycols.

Abuchowski et al., J. Biol. Chem. 252(11), p. 3578, disclose covalent attachment to an amino group of bovine serum albumin of methoxypolyethylene glycols.

U.S. Patent No. 3,619,371 discloses a polymeric matrix having a biologically active substance chemically bound thereto.

U.S. Patent No. 3,788,948 discloses use of organic cyanate compounds to bind proteins to polymers.

U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a proteolytic enzyme linked covalently to a polymeric substance.

JP 57-92435, published November 26, 1982, discloses modified polypeptides, where all or part of the amino groups are substituted with a polyethoxyl moiety. DE 2312615, published September 27, 1973, discloses conjugating of polymers to compounds containing hydroxy or amino groups.

EP 147,761 discloses a covalent conjugate of α-1-proteinase inhibitor and a water-soluble polymer, where the polymer may be polyethylene glycol.

EP 154,316, published September 11, 1985, discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of a lymphokine.

U.S. Patent No. 4,414,147 describes rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride).

PCT WO 87/00056, published January 15, 1987, discloses conjugation of PEG and polyoxyethylated polyols to such proteins as interferon-β, interleukin-2, and immunotoxins.

Davis et al., U.S. Patent No. 4,179,337, provide peptides and polypeptides coupled to polymers which are substantially non-immunogenic. In the process of Davis et al., a substantially straight-chain polymer is modified, suitably at one end thereof, either by the alteration of the terminal group or by the addition thereto of a coupling group having activity vis-a-vis polypeptide and reacting said activated polymer with the polypeptide.

Davis et al. indicate that the glycol couples most likely through an amino group on the protein, but also discloses an embodiment where the terminal hydroxy group of the glycol is converted to an amino group, e.g., with a sulfonating agent or a halogenating agent, and the resultant halide or tosylate is coupled with a carboxyl group of the polypeptide by known methods. Although the reduction of biological activity is less than where amino groups on the protein are the coupling sides, the activity is still reduced.

Summary of the Invention

It is an object of the present invention to provide a method for reducing the immunogenicity of biologically active macromolecules, while maintaining their activity.

Upon further study of the specification and appended claims, further objects and advantages of this invention will become apparent to those skilled in the art.

These objects have been satisfied by providing a process for coupling glycols to macromolecules through glycosylations on those macromolecules, instead of through amino or carboxyl groups on the macromolecule backbone itself, and by providing macromolecules having unexpectedly decreased immunogenic response and surprisingly maintained activity. Molecules in accordance with the invention, in addition, exhibit increased biological half-life, due to steric blocking of clearance receptors; increased solubility of hydrophobic molecules in an aqueous environment, due to the addition of lipophilic moieties; and increased resistance to proteolysis, due to steric hindrance.

One aspect of the invention, therefore, is a process for the glycolation of a glycosylated macromolecule, comprising activating a polyalkylene glycol, reacting the activated polyalkylene glycol with a diamino compound, whereby the activated polyalkylene glycol is coupled to the diamino compound through one of its amino groups,

oxidizing the macromolecule to activate at least one glycosyl group therein, and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule. Specifically, the invention preferably comprises a process for the PEGylation of a glycosylated macromolecule comprising:

(a) reacting a polyethylene glycol of the formula $CH_3O-(CH_2CH_2O)_n-H$ with o-nitrophenylchloroformate and triethylamine to produce a nitro compound of the formula $CH_3O-(CH_2CH_2O)_n-COO-Ph-NO_2$,

(b) reacting the nitro compound with a diaminoalkane of the formula $H_2N-(CH_2)_x-NH_2$ to produce an amino compound of the formula $CH_3O-(CH_2CH_2O)_n-CO-NH-(CH_2)_x-NH_2$,

(c) oxidizing sugar groups on the macromolecule to produce a macromolecule with an oxidized sugar residue, and

(d) reacting the amino compound with the activated macromolecule to produce a PEGylated molecule. The preferred molecular weight of the polyethylene glycol is up to about 24,000; accordingly, n is preferably about 2-500. In the diaminoalkane, x is preferably about 1-20.

The result of this preferred process is a PEGylated glycosylated macromolecule, wherein PEG is bonded to the macromolecule through its glycosylations, specifically, of the formula PEG-OCO-NH-alkylene-N=CH-macromolecule.

Macromolecules usable in the invention include virtually any bioactive macromolecule bearing glycosylations (regardless of how bonded, e.g., covalently, etc.) or which can be glycosylated, e.g., polypeptides and/or proteins, nucleic acids, lipids, or carbohydrates. Preferred peptides include those comprising an antigen binding region, a cytokine, a receptor, an antithrombotic, a growth factor, or an angiohypotensive reagent. More preferably, the polypeptide is an immunoglobulin, an interferon, a receptor tyrosine kinase, a thrombomodulin, a transforming growth factor, an endothelin, or an analog of the above. One especially preferred protein is the

monoclonal antibody TAb-250 or its chimeric analog BACh 250 ("BACh-250" or "C-erb-B2"). See Molecular Oncology as a Basis for New Strateties in Cancer Therapy: Efficacy of an Anti-c-erbB-2 Mouse/Human Chimeric Antibody Alone and in Combination with cis-Diammedichloroplatinum (CDDP), Langton et al., Proceedings of the 2nd Joint Meeting of the American Association for Cancer Research and the Japenese Cancer Association.

The procedures of the present invention are applicable to enzymes and peptide hormones. Examples of enzymes which can be used are:

oxidoreductases, such as Urate:oxygen-oxidoreductase, Hydrogen-peroxide:hydrogen-peroxide oxidoreductase, Cholesterol-reduced-NADP:oxygen oxidoreductase (20-β-hydroxylating);

transferases, such as UDP glucuronate glucuronyl-transferase (acceptor unspecific), UDP glucose:α-D-Galactose-1-phosphate;

hydrolases, such as Mucopeptide N-acetyl-muramyl-hydrolase, Trypsin, L-asparagine aminohydrolase;

lyases, such as Fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase;

isomerases, such as D-Xylose ketol-isomerase; and

ligases, such as L-Citrulline and L-aspartate ligase.

Examples of peptide hormones that can be used are insulin, ACTH, Glycagon, Somatostatin, Somatotropin, Thymosis, Parathyroid hormone, Pigmentary hormones, Somatomedin, Erythropoietin, Luteinizing hormone, Chorionic Conadotropin, Hypothalmic-releasing factors, Antidiuretic hormones, Thyroid-stimulating hormone, and Prolactin.

Macromolecules which have no sugars may be glycosylated by means which are well known in the art, e.g., as disclosed in Creighton, Proteins, W.H. Freeman & Co., New

York, 1983. Virtually any sugar which is reactive to oxidation is suitable. Examples include galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, sialic acids, fucose, and/or xylose. The length of the carbohydrate chains of the sugar may vary widely, i.e., poly- and oligosaccharides may be used. Normally, the glycosylation will be that which is indigenous to the species producing the macromolecule, e.g., mammalian.

The glycol is preferably polyethylene glycol, polypropylene glycol, or a mixture thereof, as well as a mixed polyethylene-polypropylene glycol. Polyethylene glycols (PEG's) are most preferred, especially monomethoxyethylene glycol. Preferred polyethylene glycols have the formula $CH_3O-(CH_2CH_2O)_n-H$, wherein n is 2-500, more preferably 20-400.

The diamino compound preferably has the formula $H_2N-R-NH_2$, wherein R is an organic moiety. R may be, for example, a C_{1-20}-aliphatic or C_{3-20}-cycloaliphatic moiety or a C_{5-20}-aryl moiety. Aliphatic moieties include straight- or branched-chain or cyclic alkyl, alkenyl, dienyl, and alkynyl groups. Preferred aliphatic moieties are C_{2-12}-alkyl groups. Preferred aryl moieties are heterocyclic, i.e., containing one or more O, S, or N atoms, and aromatic, e.g., phenyl groups.

Activation of the glycol with the addition of the diamino compound preferably occurs as in Veronese et al., Applied Biochemistry-Biotechnology, Vol. 11, pp. 141-152 (1985). The glycol is activated by reaction with 2,4,5-trichlorophenyl-chloroformate or p-nitrophenylchloroformate and triethylamine to yield a glycolphenylcarbonate, which is then reacted with the diamino compound in high excess so that only one amino group of the diamino compound reacts with the activated glycol. Preferably, the molar ratio of diamino compound to activated glycol is at least 2:1, and more preferably at least 10:1. The reaction proceeds rapidly for a time preferably from about 1 minute to 2 hours, more preferably from

about 5 minutes to an hour. Typically temperatures from 4°C to 100°C will be utilized, preferably between 10° and 60°, more preferably near ambient, i.e., room temperature.

Alternatively, the glycol may be activated as in Davis et al., U.S. Patent No. 4,179,337, by reacting the glycol at its terminal hydroxyl group either with a sulfonating agent, such as toluene chloride, or with a halogenating agent, such as triphenyl phosphine in carbon tetrachloride or triphenyl phosphine with a suitable N-halosuccimide. The thus-produced halide or tosylate is then treated with sodium azide and reduced with lithium aluminum hydride to give the corresponding terminal amino compound.

The activated, amino group-containing glycol is then reacted with the macromolecule, on which a carbohydrate, i.e., a sugar moiety, has been oxidized. Preferably, oxidation is accomplished by reaction of the macromolecule with sodium periodate ($NaIO_4$) in a preferred molar ratio of sugar to $NaIO_4$ of 1000:1 to 10:1. The concentration of $NaIO_4$ is preferably 10-1000 mM. The reaction may preferably proceed at temperatures of 0°C to 50°C, and preferably for 1 minute to 4 hours, more preferably about 30 minutes to 2 hours, most preferably about 30 minutes. Adjustment of the reaction time may be made to control the amount of glycols per protein, since longer incubation results in greater oxidation of the sugars and, accordingly, more points on the macromolecule available for glycol attachment. Care should be taken to avoid over-glycolation, resulting in stearic hinderance of the macromolecule, reducing efficient reaction with its intended target. One of ordinary skill in the art could easily, with only routine experimentation, optimize this portion of the process for an intended use of the macromolecule.

Following oxidation of the sugars, the macromolecule is coupled with the amino group-bearing glycol, by simple

mixing, at temperatures preferably of about 4°C to 100°C, and for times preferably from about 1 minute to 5 hours, more typically between 3 minutes and 1 hour, and more preferably between 5 minutes and 30 minutes. Isolation, if desired, and work-up for biological applications is conventional, as disclosed in, e.g., U.S. Patent No. 4,179,337 or as described below. The macromolecules may be used as diagnostic reagents, therapeutic reagents, test samples, etc., as known in the art and dependent on their disclosed biological utilities.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative and not limitative of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

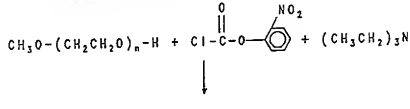
The entire disclosure of all applications, patents and publications, cited above and below, are hereby incorporated by reference.

EXAMPLES

1. Activation of Methoxy-PEG (mPEG)

Two grams of mPEG (0.1 mM, final concentration), 15 Kd, is dissolved in 20 ml of acetonitrile with 0.24 g of o-nitrophenylchloroformate (1.2 mM) and 33 µl of triethylamine (1.2 mM) and stirred for 24 hours at room temperature.

Chemical Reaction



mPEG-µ-pNP

The triethylammonium chloride is then filtered off using a sintered glass funnel. 200 ml of ethyl ether is added, and the solution is left to crystallize overnight at 4°C. The product is filtered, washed with ether to remove all of the yellow color, and recrystallized from acetonitrile-ether. The yield is 75%. The product is then assayed spectrophotometrically by the release of p-nitrophenol by ε-amino-n-caproic acid (ACA).

The purity of the product is further verified spectrophotometrically.

2. PEGylation of TAb-250 Through Lysine Groups

Protocol

5 mg of TAb-250 (0.0316 µmoles) is dialyzed extensively into 50 mM sodium borate buffer pH 8.3. A lower pH is used in order to ensure that only the very reactive epsilon amino groups of lysine are PEGylated.

To the 2 ml dialyzed sample 3 mg of the activated mPEG is added, a 5 molar excess. Every 30 minutes, 5.μl is removed and mixed with 5 μl of 25 mM ACA. Immediately, 3 mg of activated mPEG is added and incubated at room temperature with shaking for a further 30 minutes. The reaction is stopped after 2 hours; final molar excess is 20-fold, by loading the sample on a NAP 25 (Pharmacia) desalting column and eluting it with 50 mM $NaPO_4$ buffer, pH 6.8. The desalted sample is loaded on Superose 6 column (1 x 30 cm BioRad Econocolumn®) and eluted with 50 mM $NaPO_4$ buffer, pH 6.8. Four resultant peaks from the Superose column and the 30-minute time point samples are assayed by SDS-PAGE. The Superose peaks are further assayed by Radial Immuno-diffusion (RID) from Tago Immuno, Inc. for the quantitation of mouse IgG_1, more specifically a kappa light chain. See Table 1.

Half of the pooled superose b sample is PEGylated as described above; however, this time, activated PEG was in 100x molar excess and incubated for 3 hours. Half of this sample is allowed to incubate overnight before being stopped.

All samples were processed and assayed as reported above.

Chemistry:

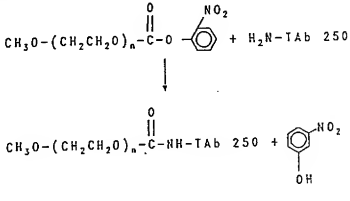


TABLE 1

Sample	Diameter	µg/mL	Est. Binding Efficiency %
PEG-µ-butamine TAb 250	11.6	Out of range	100
PEG-µ-TAb 250 100x excess	5.6	25	16.7
TAb 250 in borate pH 8.3	6.8	150	100
PEG-µ-TAb 250 20x (peaks 1+2)	7.7	129	100
Oxidized TAb 250	5.3	51.3	100
Tago Standards IgG Tago Immuno, Inc.	5.5	0.16/mg/mL (Conc. on vial = 0.156 mg/mL)	

3. **PEGylation of TAb-250 Through the Carbohydrate Moieties**

Immunoglobulin G (IgG_1) contains approximately 3% carbohydrate by weight linked to the F_c region of the protein.

Protocols

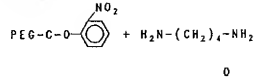
(a) **Making the amino derivative of mPEG-µ-pNP**

0.5 g of mPEG-µ-p-nitrophenyl is slowly added to 5 mL of 50 mM Na-borate buffer, pH 9.0, containing 44.25 mg (100 mmoles) of 1,4-aminobutane. The reaction is incubated at room temperature with shaking for 3 hours. The reaction is stopped by passing it through an NAP 25 desalting column and eluted with water and dialyzed into milli-Q H_2O.

The dialyzed material is lypophylized and weighed.

Total yield of PEG-µ-butamine = 0.573 g.

Chemistry



(PEG-μ-butamine)

During the reaction, 1,4-diaminobutane is in high excess to guard against reacting both amino groups.

(b) Oxidation of TAb-250

Immunoglobulin G_1 (IgG_1) contains approximately 3% carbohydrate by weight linked to the F_c region of the protein.

Coupling Buffer:	0.05 M sodium acetate 0.1 M sodium chloride, pH 5.0
Wash Buffer:	0.1 M sodium acetate 0.5 M sodium chloride, pH 3.5
Storage buffer:	0.05 M sodium phosphate, pH 6.8

0.5 mg of TAb-250 is buffer exchanged into the coupling buffer using an NAP-10 (Pharmacia) desalting column. To the TAb-250 solution is added 0.1 mL of freshly prepared 100 mM sodium m-periodate ($NaIO_4$). The solution is mixed gently, and the sealed reaction vial is shielded from light and incubated at room temperature for 30 minutes. To stop the reaction, the sample is passed through a NAP-10 desalting column and is equilibrated with wash buffer. The column is eluted with the conjugation buffer.

(c) Coupling of oxidized TAb-250 to PEG-μ-butamine

To the desalted, oxidized TAb-250 is added 5 mg of PEG-μ-butamine. The reaction vial is overlayed with nitrogen and is tumbled gently overnight at 4°C. The

molar ratio of TAb-250 to PEG-μ-butamine is 1:100. The sample is then loaded following optional reduction of the TAb-250 onto the same Superose 6 column. The IgG peaks are pooled and are concentrated on an amicon stirred cell concentrator.

All the pegylated TAb 250 species were further analyzed by SDS-PAGE so as to estimate their molecular weights.

Discussion

From the SDS-PAGE gel analysis, it can be seen that the experiments have generated species of a higher molecular weight than TAb-250. Table 1 demonstrates that 100x molar excess sample has lost most of its binding capacity, as expected. It would indicate that all of the lysine binding sites are saturated, thus hindering binding. The 20x molar PEG excess and the carbohydrate conjugated samples all appear to retain all of their binding capacity.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. A glycolated, glycosylated macromolecule, wherein a glycol is bonded to the macromolecule through a glycosylation moiety.

2. A glycolated, glycosylated macromolecule according to claim 1, wherein the glycol is a polyalkylene glycol.

3. A glycolated, glycosylated macromolecule according to claim 2, wherein the glycol is a polyethylene glycol.

4. A glycolated, glycosylated macromolecule according to claim 1 of the formula:

glycol-diamine-macromolecule,

wherein the diamine is bonded to the macromolecule through a carbohydrate moiety thereof, thereby forming a Schiff base linkage.

5. A glycolated, glycosylated macromolecule according to claim 1 of the formula:

glycol-OCO-NH-alkylene-N=CH-macromolecule,

wherein "alkylene" has 1 to 20 carbon atoms.

6. A glycolated, glycosylated macromolecule according to claim 1, which is a pharmacologically active compound.

7. A glycolated, glycosylated macromolecule according to claim 1, wherein said macromolecule comprises a nucleic acid, a polypeptide, or a lipid.

8. A glycolated, glycosylated macromolecule according to claim 7, wherein the polypeptide is a protein.

9. A glycolated, glycosylated macromolecule according to claim 8, wherein the protein is TAb-250 or BACh-250.

10. A glycolated, glycosylated macromolecule according to claim 1, having essentially undiminished bioactive half-life in a host and a reduced immunogenic side effect, an increased aqueous solubility, an increased resistance to proteolytic digestion, or a decreased affinity for formulation polymers, as compared to said glycosylated macromolecule which is not glycolated.

11. A glycolated, glycosylated macromolecule according to claim 1, having increased bioactive half-life compared to a glycosylated macromolecule which is not glycolated.

12. A glycolated, glycosylated macromolecule according to claim 1, having essentially undiminished bioactive half-life in a host, as compared to said glycosylated macromolecule which is glycolated through amino or carboxyl groups not on the glycosyl portion of the macromolecule.

13. A glycolated, glycosylated macromolecule according to claim 7, wherein said macromolecule is a polypeptide which comprises an antigen binding region.

14. A glycolated, glycosylated macromolecule according to claim 7, wherein said macromolecule is a polypeptide which is a cytokine, a receptor, an anti-thrombotic, a growth factor, or an angiohypotensive reagent.

15. A glycolated, glycosylated macromolecule according to claim 7, wherein said polypeptide is an immunoglobulin, an interferon, a receptor tyrosine kinase, a thrombomodulin, a transforming growth factor, or an endothelin.

16. A process for the reduction of immunogenic side effect, for increasing aqueous solubility, for increasing resistance to proteolytic digestion, or for increasing the half-life of a bioactive glycosylated macromolecule upon administration to a host, comprising glycolating said macromolecule by bonding glycol to a carbohydrate moiety thereof.

17. A process according to claim 16, wherein the glycol is bonded to the macromolecule by a Schiff base linkage.

18. A process according to claim 16, wherein the macromolecule is a polypeptide.

19. A process according to claim 18, wherein the polypeptide is a protein.

20. A process according to claim 19, wherein the protein is TAb-250 or BACh-250.

21. A process for the glycolation of a glycosylated macromolecule, comprising activating a polyalkylene glycol, reacting the activated polyalkylene glycol with a diamino compound whereby the activated polyalkylene

glycol is coupled to the diamino compound through one of its amino groups, oxidizing the macromolecule to activate at least one glycosyl group therein, and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule.

22. A method of claim 21, wherein said activating of polyalkylene glycol produces a glycol-nitrophenyl carbonate intermediate.

23. A process according to claim 21, wherein said diamino compound has the formula $H_2N-R-NH_2$, wherein R is an organic moiety.

24. A process according to claim 23, wherein R is an aliphatic hydrocarbon having from 2 to 20 carbon atoms or an aromatic hydrocarbon having from 5 to 20 carbon atoms.

25. A process according to claim 24, wherein R is an aliphatic hydrocarbon having from 5-12 carbon atoms.

26. A process according to claim 21 for the PEGylation of a glycosylated macromolecule comprising:

(a) reacting a polyethylene glycol of the formula

$$CH_3O-(CH_2CH_2O)_n-H$$

with o-nitrophenylchloroformate and triethylamine to produce a nitro compound of the formula

$$CH_3O-(CH_2CH_2O)_n-COO-Ph-NO_2,$$

(b) reacting the nitro compound with a diaminoalkane of the formula

$$H_2N-(CH_2)_x-NH_2$$

to produce an amino compound of the formula

$$CH_3O-(CH_2CH_2O)_n-CO-NH-(CH_2)_x-NH_2,$$

(c) oxidizing sugar groups on the macromolecule to produce a macromolecule with an oxidized sugar residue, and

(d) reacting the amino compound with the macromolecule to produce a PEGylated molecule, wherein n is 2-500 and x is 1-20.

27. A process according to claim 26, wherein n is 20-400 and x is 2-12.

28. A process according to claim 21, wherein the macromolecule is a nucleic acid, a polypeptide, or a lipid.

29. A process according to claim 21, wherein the macromolecule is a pharmacologically active compound.

30. A glycolated, glycosylated macromolecule produced by a process of claim 16.

31. A glycolated, glycosylated macromolecule produced by a process of claim 21.

32. A PEGylated, glycosylated macromolecule produced by a process of claim 26.